



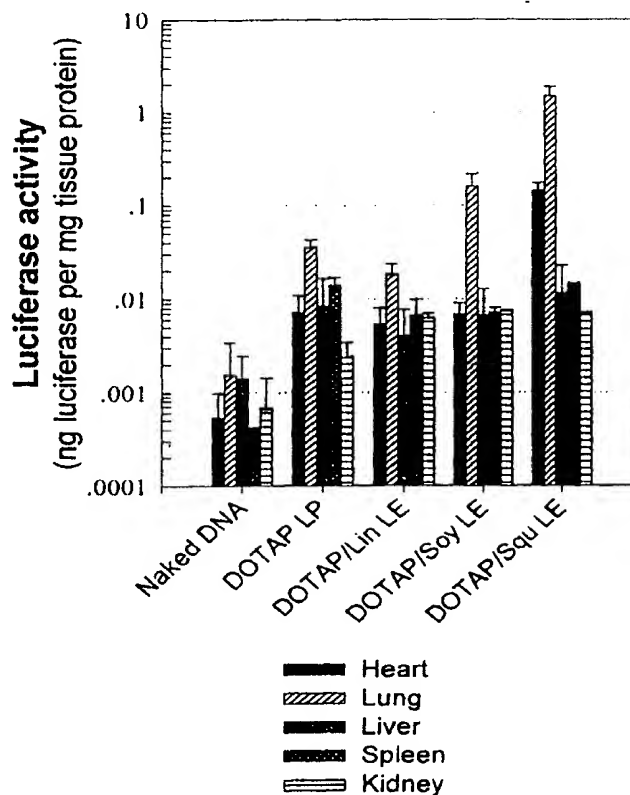
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>A61K 9/107</b>	<b>A1</b>	(11) International Publication Number: <b>WO 00/06120</b> (43) International Publication Date: 10 February 2000 (10.02.00)
<p>(21) International Application Number: PCT/KR99/00414</p> <p>(22) International Filing Date: 30 July 1999 (30.07.99)</p> <p>(30) Priority Data: 1998/31249 31 July 1998 (31.07.98) KR</p> <p>(71) Applicant (for all designated States except US): KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY [KR/KR]; 39-1, Hawolgok-Dong, Sungbook-Ku, Seoul 136-791 (KR).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): JEONG, Seo, Young [KR/KR]; 5, Munchonmaeul Life Apt. 205/501, Juyeop 2-Dong, Ilsan-Ku, Koyang, Kyungki-Do 411-372 (KR). KWON, Ick, Chan [KR/KR]; 274, Shiyong Apt. 706-704, Hakye-Dong, Nowon-Ku, Seoul 130-230 (KR). CHUNG, Hesson [KR/KR]; Jukong Apt. 201-1005, Mansoo-Dong, Namdong-Ku, Incheon 405-240 (KR).</p> <p>(74) Agent: PARK, Jang, Won; Park, Kim &amp; Partner, Jewoo Bldg. 4th Fl. 200, Nonhyun-Dong, Kangnam-Ku, Seoul 135-010 (KR).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>

(54) Title: LIPID EMULSION AND SOLID LIPID NANOPARTICLE AS A GENE OR DRUG CARRIER

## (57) Abstract

The present invention relates to oil-in-water lipid emulsions composed of non-triglyceride oils and solid lipid nanoparticles (SLN) composed of triglyceride or ethyl stearate used as gene transfection agents and drug delivery systems and method for preparing thereof. The present invention also concerns the method of transferring genes or drugs efficiently into cells by using the lipid emulsions and solid lipid nanoparticles. Also the present invention relates to the method of preparing lipid emulsions containing lipophilic or amphiphilic drugs by using squalene or squalane as the core-oil. The present invention also concerns the method of preparing the solid lipid nanoparticles containing lipophilic or amphiphilic drugs by using ethyl stearate as the core-fat.



***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**LIPID EMULSION AND SOLID LIPID NANOPARTICLE**  
**AS A GENE OR DRUG CARRIER**

**DISCLOSURE OF THE INVENTION**

5

The present invention relates to oil-in-water lipid emulsions, a complex between the emulsion and genes, and emulsions loaded with hydrophobic or amphiphilic drugs using biocompatible and biodegradable fats and oils. The present invention also relates to a method of preparing oil-in-water lipid emulsions, a  
10 complex between the emulsion and genes, and emulsions loaded with hydrophobic or amphiphilic drugs using biocompatible and biodegradable fats and oils.

The present invention also relates to a method of transferring genes efficiently into cells by using said lipid emulsions.

15

**BRIEF DESCRIPTION OF THE DRAWINGS**

The present invention will become more fully understood from the detailed description given hereinbelow and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and  
20 wherein:

Figure 1 is a graph showing a correlation between the average droplet size of the lipid emulsion for different oils and the interfacial tension between water and oil;

Figure 2a is a graph showing a correlation between the average droplet size of the lipid emulsion using different triglycerides and the concentration of DLPC;

25 Figure 2b is a graph showing a correlation between the changes in the average droplet size of the lipid emulsion using tributyrin and the concentration of DLPC;

Figure 2c is a graph showing a correlation between the changes in the average droplet size of the lipid emulsion using tricaproin and the concentration of  
30 DLPC;

Figure 2d is a graph showing a correlation between the changes in the average droplet size of the lipid emulsion using tricaprylin and the concentration of DLPC;

Figure 3a is a graph showing a correlation between the average droplet size

of the lipid emulsion using tributyrin and the concentration of the emulsifiers;

Figure 3b is a graph showing a correlation between the average droplet size of the lipid emulsion using tricaproin and the concentration of the emulsifiers;

Figure 3c is a graph showing a correlation between the average droplet size  
5 of the lipid emulsion using tricaprylin and the concentration of the emulsifiers;

Figure 4 is an electrophoresis photograph showing a complex formation between DNA and lipid emulsion of the present invention. One microgram of pCMV-beta plasmid and different amounts of lipid emulsion were used to form a complex;

10 Lane 1: DNA molecular weight marker;

Lane 2: 1  $\mu$ g of pCMV-beta plasmid [7164 base pairs (bp)];

Lane 3: 2  $\mu$ l of Lipofectamine and 1  $\mu$ g of pCMV-beta plasmid;

Lane 4: 4  $\mu$ g of DOTAP liposome and 1  $\mu$ g of pCMV-beta plasmid;

15 Lanes 5, 6, 7 and 8: 1, 2, 4 and 6  $\mu$ g, respectively, of DOTAP/squalene lipid emulsion and 1  $\mu$ g of pCMV-beta plasmid;

Lane 9: 4  $\mu$ g of DOTAP/soybean oil lipid emulsion and 1  $\mu$ g of pCMV-beta plasmid;

Lane 10: 4  $\mu$ g of DOTAP/linseed oil lipid emulsion and 1  $\mu$ g of pCMV-beta plasmid.

Figure 5 is an electrophoresis photograph of pCMV-beta after the carrier/DNA complexes underwent an exchange reaction with poly-L-aspartic acid;

20 A) Lipofectamine and DOTAP liposome, B) linseed oil emulsion/pCMV-beta complex, C) soybean oil emulsion/pCMV-beta complex and D) squalene emulsion/pCMV-beta complex;

Lane 1: DNA molecular weight marker;

Lane 2: 1  $\mu$ g of pCMV-beta plasmid [7164 base pairs (bp)];

25 Lane 3: carrier/ pCMV-beta complex;

Lanes 4-13: carrier/ pCMV-beta complex incubated with 0.625, 1.25, 2.5, 5.0, 25, 50, 100, 200, 400 and 800 equivalency of poly-L-aspartic acid for an hour. Equivalency in the present invention represents the charge ratio between phosphate group of DNA and carboxylic group of PLAA.

30 Figure 6 is a transmission electron micrograph of cationic solid lipid nanoparticle, DNA and their complex.

A) pCMV-beta, B) trilaurin solid lipid nanoparticle, C) complex between trilaurin solid lipid nanoparticle and pCMV-beta (1/1 by weight) and D) complex between trilaurin solid lipid nanoparticle and pCMV-beta (2/1 by weight).

B)

Figure 7 is a graph showing the effect of a helper lipid DOPE on the transfection efficiency of liposome and emulsion carriers;

A) DOTAP/DOPE liposome, B) DOTAP/DOPE squalene lipid emulsion

5 ■ : no serum, □ : 80 % serum.

Figure 8 is a graph showing the effect of a helper lipid diolein on the transfection efficiency of liposome and emulsion carriers;

A) DOTAP/DOPE liposome, B) DOTAP/DOPE squalene lipid emulsion

■ : no serum, □ : 80 % serum.

10 Figure 9 is a graph showing the changes in the transfection efficiency of liposome and emulsion carriers by adding a non-ionic surfactant, Tween 80;

A) DOTAP/DOPE/Tween 80 liposome, B) DOTAP/DOPE/Tween 80 squalene lipid emulsion

■ : no serum, □ : 80 % serum.

15 Figure 10 is a graph showing the stability of the lipid emulsion by adding Tween 80;

$\mu$  : DOTAP/DOPE squalene lipid emulsion,  $\lambda$  : DOTAP/DOPE/Tween 80 squalene lipid emulsion.

Figure 11 is a graph showing the changes in the transfection efficiency using the lipid carriers by adding protamine sulfate;

20 A) protamine sulfate B) 4  $\mu$ g of DOTAP/DOPE/Tween80 liposome and protamine sulfate C) 4  $\mu$ g of DOTAP/DOPE/Tween80 lipid emulsion and protamine sulfate

$\lambda$  : no serum,  $\mu$  : 80 % serum.

Figure 12 is a graph showing the transfection efficiency using different lipid gene carriers with various cell-lines

25 ■ : no serum, □ : 80 % serum.

Figure 13 is a graph showing the difference in *in vitro* release rates of rifampicin from different lipid emulsions;

$\nabla$  : PBS,  $\lambda$  : linseed oil emulsion,  $\mu$  : soybean oil emulsion,  $\tau$  : squalene emulsion.

30 Figure 14 is a graph showing the difference in *in vitro* release rates of diclofenac from different lipid emulsions;

$\nabla$  : PBS,  $\lambda$  : linseed oil emulsion,  $\mu$  : soybean oil emulsion,  $\tau$  : squalene emulsion.

Figure 15 is a graph comparing the *in vivo* transfection efficiencies when 10

μg of DNA was administered intravenously by using different lipid gene carriers.

Figure 16 is a graph comparing the *in vivo* transfection efficiencies when 50 μg of DNA was administered intravenously by using different lipid gene carriers.

Figure 17 is a graph comparing the *in vivo* transfection efficiencies when DNA was administered intravenously by using different lipid gene carriers: effect of emulsifiers with PEG moiety.

Figure 18 is a graph comparing the *in vivo* transfection efficiencies when DNA was administered intravenously by using different lipid gene carriers: effect of protamine sulfate.

Figure 19 is a graph comparing the *in vivo* transfection efficiencies when DNA was administered by intranasal instillation by using different lipid gene carriers.

Figure 20 is a graph showing the difference in the *in vitro* release rates of diclofenamic acid from different lipid emulsions;

▽ : PBS, λ : linseed oil emulsion, μ : soybean oil emulsion, τ : squalene emulsion.

15

## **TECHNICAL FIELD**

It is an object of the present invention to provide a method of preparing oil-in-water type lipid emulsion and solid lipid nanoparticles composed of biocompatible materials.

Another object of the present invention is to provide a complex between biologically active material and lipid emulsion comprising non-triglyceride oils and a complex between biologically active material and solid lipid nanoparticle comprising a fat selected from the group comprising triglycerides and ethyl stearate, and the preparation method thereof.

Another object of the present invention is to provide lipid emulsions comprising squalene or squalane loaded with drugs or to provide ethyl stearate solid lipid nanoparticles loaded with drugs and the preparation methods thereof.

The present invention also concerns the method of transferring genes and drugs efficiently into cells by using the lipid emulsions.

## **BACKGROUND ART**

Liposome and emulsion are the two of the well-known non-viral gene carriers.

Gene therapy using liposomes are under clinical trials. There are many reports in the past several years regarding liposome-mediated gene delivery.

Triglyceride lipid emulsion, which has been developed recently, may form a complex that maintains physical stability and delivers genes in the presence of serum. These triglyceride emulsions, however, has a low physical stability and loses the ability to transfer genes without a polymeric lipid such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-N[-poly(ethyleneglycol)2000](PEG<sub>2000</sub>PE). The polymeric lipids that may provide physical stability of emulsions include Span, Tween, and Brij series lipids having polyethylene glycol moiety in the lipid headgroup. These polymeric lipids, however, may attenuate the interaction between the lipid emulsion and the gene since the polymeric moiety provides a steric hindrance. Therefore, the cationic lipid emulsion containing polymeric lipid could form a complex with DNA when there are 3-4 excess positive charges of emulsions.

To overcome these problems, an object of the present invention is to prepare a stable lipid emulsion without or with a minimal amount of polymeric lipid. Accordingly, we have used different oils that are known to be biocompatible with an acceptable toxicity. Among the lipid emulsions when egg phosphatidylcholine (PC) was used as an emulsifier, we have discovered that the oils that have higher interfacial tension against water forms more stable emulsions than those with a lower interfacial tension. We also have discovered that the correlation between emulsion stability and the oil/water (o/w) interfacial tension has not been systemically studied. There is a small indication that the oils that have smaller o/w interfacial tension may form a more stable emulsion. Therefore, the present inventors tried to provide a scientific background to provide a reason for the correlation between the emulsion stability and the physical properties of oils.

***Preliminary experiment to provide a scientific background for the present invention:***

Lipid emulsions are generally prepared by dispersing oil in water by use of an emulsifier. There are many theoretical works on how emulsions can be stabilized. While studying the factors to stabilize the lipid emulsions, the present inventors have found that there are only few studies on the effect of the oils that have different physical properties such as lipophilicity and the stability of the resulting emulsions.

To investigate how the lipophilicity of the oils changes the stability of the o/w

emulsions, many different oils were selected to form emulsions by using egg PC as an emulsifier. The lipid emulsions having 10 %(v/v) of oils and 1.2 %(w/v) of egg PC were obtained by 1) preparing a liposome solution by sonicating egg PC in water and 2) adding oil in the liposome solution to sonicate the mixture for 2 min for three  
5 times (total 6 min). The size of the particles in the emulsion was measured by photon correlation spectroscopy. The emulsion made with linseed oil has the biggest emulsion droplet sizes whereas the squalene and jojoba bean oil formed emulsions with the smallest droplet sizes. The relationship between the size of the emulsion and the o/w interfacial tension of the oils is shown in Figure 1. The results  
10 show that the oil that has bigger o/w interfacial tension forms an emulsion with smaller droplet size. The oils are mainly mixtures of triglycerides and esters of fatty acid/alcohols, except squalene.

In the present invention, emulsions made with squalene or squalane are stable without the use of polymeric lipids. There are some examples in the previous  
15 studies on the squalene emulsions. The squalene emulsions are used as vaccine adjuvants (US patent 5,376,369). Squalene was selected as an oil phase in o/w emulsion since it has a low cytotoxicity and is biodegradable. Squalene emulsion was also formulated as a Taxol carrier (US patent 5,407,683). However, this Taxol-loaded squalene emulsion is formulated by forming a self-emulsifying glass by  
20 adding surfactant or alcohols and by dispersing it in water. A large amount of ethanol is included in the formulation.

Therefore, the composition and the preparation method are completely different from those of the present invention. In summary, squalene emulsions known up to now are 1) vaccine adjuvants to deliver antigens and 2) Taxol-loaded  
25 emulsions having different composition and preparation method from the present invention. Therefore, the lipid emulsion of the present invention to deliver genes and lipophilic or amphiphilic drugs differs from the pre-existing squalene emulsion formulas. Squalene emulsion shows a far superior stability to the emulsions made by castor, soybean, and safflower or sunflower oil. The release rate of lipophilic drug is  
30 slower from more stable emulsions. Also, bioavailability of the drug is better for more stable emulsions. Though further study is required to explain how the interfacial tension and the emulsion droplet size is related, the decrease in the interfacial tension by offering different tension gradient by different oils may be a possible explanation.



The present invention is also related to the ethyl stearate solid lipid nanoparticles. Solid lipid nanoparticles made with ethyl stearate, or more broadly the ethyl esters of fatty acid or alcohols with C10-18 straight chains, have never been used as gene carriers. Moreover, the ethyl stearate solid lipid nanoparticles have  
5 not been used as a carrier of a lipophilic or amphiphilic drug.

The present invention provides the lipid emulsion made of non-triglycerides including squalene or squalane and 1) cationic emulsifier and 2) phospholipids and lipid with PEG moiety, and the preparation methods thereof. Also, the present invention provides cationic solid lipid nanoparticles to deliver genes or other  
10 biologically active materials and the preparation method thereof. The solid lipid nanoparticles of the present invention are suspension of solid fat particles in water. Therefore, it may be freeze-dried to remove water from the system to preserve the fat particles. It has an advantage of preparing drug-loaded solid lipid nanoparticles that can be freeze-dried to increase storage time.

15 Cationic solid lipid nanoparticle is composed of triglyceride, having C12-18 straight chain hydrophobic tail, which exists as a solid at room and body temperatures and cationic emulsifier. There are no current reports on the positively charged solid lipid nanoparticle as a gene carrier.

Lipid emulsions made of triglycerides have been widely used as a drug  
20 delivery system. For instance, there are many different emulsion formulations for cyclosporin, an oligopeptide immunosuppressant. Medium chain triglycerides with C8-12 carbons were used to solubilize cyclosporin in o/w emulsions (US patent 5,660,858). Emulsions, made by alcohol, alkanol, polysorbate 80 and cyclosporin with submicron particle sizes, are reported to be stable and have high bioavailability  
25 (WO 97/35,603). According to WO 97/36,610, cyclosporin dissolved in medium chain triglycerides was dispersed in water easily by using propylene carbonate as a co-emulsifier with an average droplet size of ca. 100 nm. Although there are many cyclosporin formulations, there are no reports or patents on the emulsion formulation using squalene or squalane, highly pure branched hydrocarbons. Also, there are  
30 no reports on the cyclosporin formulation using solid lipid nanoparticles made of ethyl stearate. Squalene, squalane and ethyl stearate are mono-component oil systems. Therefore, unlike vegetable oils made of many components, the physical and chemical properties do not vary. Mono-component system provides a better quality control in mass-production. Moreover, according to the present invention, squalene

emulsion is more stable having smaller average particle sizes than many other vegetable emulsions. The in vitro drug release experiment also shows that the release of drugs such as rifampicin is sustained with a zero-order release pattern from the squalene emulsion.

- 5           The squalene and squalane emulsions and the ethyl stearate solid lipid nanoparticle can be used as a carrier of negatively-charged biologically active materials including DNA when cationic emulsifier was used and as a drug delivery system for lipophilic or amphiphilic drugs. Moreover, the cationic emulsion or solid lipid nanoparticle, loaded with lipophilic anticancer drugs can form a complex with a  
10 therapeutic gene in cancer therapy.

### **MODE(S) FOR CARRYING OUT THE INVENTION**

- According to the first aspect of the present invention, the present invention  
15 provides a lipid emulsion to deliver genes or other biologically active materials comprising a) 2-30 % of oil of non-triglycerides, b) 0.01-20 % of one or more emulsifiers including a cationic surfactant and c) water to 100 %. The lipid emulsion of the present invention can include other additives. The emulsifier in the present invention is required to make the surface charge of the emulsion positive and  
20 therefore, it is selected from an emulsifier having a positive charge to form a complex with a negatively charged biologically active material including DNA. A non-ionic surfactant, phospholipid, fatty acid, fatty alcohol, bile salt or cholesterol may be additionally used as an emulsifier.

- According to the second aspect of the present invention, the present invention  
25 provides a solid lipid nanoparticle to deliver genes or other biologically active materials comprising a) 2-30 % of triglycerides having 10-18 carbons in each hydrophobic tails or ethyl stearate, b) 0.01-20 % of one or more emulsifiers including a cationic surfactant and c) water to 100 %. The lipid emulsion of the present invention can include other additives. The emulsifier in the present invention is  
30 required to make the surface charge of the emulsion positive and therefore, it is selected from an emulsifier having a positive charge to form a complex with a negatively charged biologically active material including DNA. A non-ionic surfactant, phospholipid, fatty acid, fatty alcohol, bile salt or cholesterol may be additionally used as an emulsifier.

According to the third aspect of the present invention, the present invention provides a drug-loaded lipid emulsion to deliver a lipophilic or amphiphilic drug comprising a) 2-30 % non-triglycerides oil such as squalene or squalane, b) 0.01-20 % of one or more emulsifiers, c) 0.1-10 % lipophilic or amphiphilic drug and d) 5 water to 100 %. The drug-containing emulsion of the present invention can include other additives. The surface charge of the drug-containing emulsion of the present invention may be positive, zero or negative.

According to the forth aspect of the present invention, the present invention provides a drug-loaded solid lipid nanoparticle to deliver a lipophilic or amphiphilic 10 drug comprising a) 2-30 % of ethyl stearate, b) 0.01-20 % of one or more emulsifiers, c) 0.1-10 % lipophilic or amphiphilic drug and d) water to 100 %. The drug-containing solid lipid nanoparticle of the present invention can include other additives. The surface charge of the drug-containing solid lipid nanoparticle of the present invention may be positive, zero or negative.

15 The present invention is also related to the method of preparing the lipid emulsion to deliver gene or other biologically active materials to cells. The preparation method of the lipid emulsion according to the present invention include a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers including a cationic emulsifier and b) a second step of preparing emulsion 20 by mixing said aqueous phase with 2-30 % of non-triglycerides oil.

The present invention is also related to a method of preparing the solid lipid nanoparticle to deliver gene or other biologically active materials to cells. The preparation method of the solid lipid nanoparticle according to the present invention include a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or 25 more emulsifiers including a cationic emulsifier and b) a second step of preparing emulsion by mixing said aqueous phase with 2-30 % of fat of triglycerides having 10-18 carbons in each hydrophobic tails or ethyl stearate.

The present invention is also related to a method of preparing the lipid emulsion to deliver lipophilic or amphiphilic drugs to cells. The preparation method 30 of the drug-loaded lipid emulsion according to the present invention include a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers, b) a second step of preparing oil phase by mixing 0.1-10 % of lipophilic or amphiphilic drug and 2-30 % of non-triglycerides oil and b) a third step of preparing drug-loaded emulsion by mixing the aqueous and oil phases prepared in the first and

second steps, respectively.

The present invention is also related to a method of preparing the solid lipid nanoparticle to deliver lipophilic or amphiphilic drugs to cells. The preparation method of the drug-loaded solid lipid nanoparticle according to the present invention include a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers, b) a second step of mixing 0.1-10 % of lipophilic or amphiphilic drug and 2-30 % of fat such as ethyl stearate and b) a third step of preparing drug-loaded solid lipid nanoparticle by mixing the aqueous and oil phases prepared in first and second steps, respectively.

It is also possible to prepare a drug-loaded lipid emulsion or solid lipid nanoparticle by first preparing an oil or fat phase, respectively, comprising oil or fat, respectively, emulsifier and drugs and subsequently by mixing the oil phase with water.

Also, the present invention is related to the preparation method of the lipid emulsion to deliver biologically active materials including genes to cells. The preparation method includes a) a first step of preparing an oil phase by mixing 2-30 % of one or more emulsifier including a cationic emulsifier with 0.1-20 % of non-triglycerides oil and b) a second step of mixing the oil phase with water.

The present invention is also related to the preparation method of the solid lipid nanoparticle to deliver biologically active materials including genes to cells. The preparation method includes a) a first step of preparing a fat phase by mixing 0.1-20 % of one or more emulsifier including a cationic emulsifier with 2-30 % fat of triglycerides having 10-18 carbons in each hydrophobic tails and ethyl stearate and b) a second step of mixing the fat phase with water.

Also the present invention is related to a method of preparing the drug-loaded lipid emulsion. The preparation method includes a) a first step of preparing an oil phase by mixing 0.1-20 % of one or more emulsifier and 0.1-10 % of lipophilic or amphiphilic drug with 2-30 % of non-triglycerides oil and b) a second step of mixing the oil phase with water.

The present invention is also related to a method of preparing the drug-loaded solid lipid nanoparticle. The preparation method includes a) a first step of preparing an fat phase by mixing 0.1-20 % of one or more emulsifier and 0.1-10 % of lipophilic or amphiphilic drug with 2-30 % fat such as ethyl stearate and b) a second step of mixing the oil phase with water.

The lipid emulsion according to the present invention represents a heterogeneous mixture of two or more immiscible liquid stabilized by the use of a surfactant or an emulsifier.

The solid lipid nanoparticle according to the present invention represents a solid-state fat dispersed in a liquid stabilized by the use of a surfactant or an emulsifier.

The non-triglycerides of the present invention include squalene and squalane.

The fat in the solid lipid nanoparticle of the present invention includes ethyl ester of alcohol or acid having a straight chain having 10-18 carbons, and preferably ethyl stearate.

The emulsifier according to the present invention may additionally include phospholipid or non-ionic surfactant. The cationic surfactant used as emulsifiers of the present invention include 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2-distearoyl-3-trimethylammonium-propane (DSTAP), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dimyristoyl-3-dimethylammonium-propane (DMDAP), 1,2-dipalmitoyl-3-dimethylammonium-propane (DPDAP), 1,2-dilauroyl-3-dimethylammonium-propane (DLDAP), 1,2-distearoyl-3-dimethylammonium-propane (DSDAP), dimethyldioctadecylammonium chloride (DDAB), N-[1-(1,2-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 1,2-dioleoyl-3-ethylphosphocholine (DOEPC) and other cationic phospholipid. The cationic emulsifiers are required to deliver genes since they may not only interact with negatively charged DNA to form a complex but also enhance the interaction with cells with an excess positive charge.

The phospholipids according to the present invention include phosphatidylcholine (PC) and derivatives thereof, phosphatidylethanolamine (PE) and derivatives thereof and phosphatidylserine (PS). Among the phospholipids, L- $\alpha$ -dioleoyl phosphatidylethanolamine (DOPE) is well known to disturb the endosomal membrane allowing DNA to enter cytoplasm and as a consequence to increase the transfection efficiency.

A fusogenic lipid including DOPE or diolein may be additionally used as an emulsifier. Also, fatty acid, fatty alcohol, cholesterol or bile salt may be additionally used as an emulsifier to increase the physical stability of the lipid emulsion or solid lipid nanoparticle.

Non-ionic surfactants in the present invention include poloxamers (also known as a pluronic: a copolymer of polyoxyethylene and polyoxypropylene), sorbitan esters (Span), polyoxyethylene-sorbitan fatty acid esters (Tween) and polyoxyethylene ethers (Brij).

5        Additionally, the present invention may comprise hydrophilic polymers, polymeric lipid where the hydrophilic polymers are covalently bonded to a phospholipid or polymerizable lipid such as diacetylated phospholipid. Examples of the hydrophilic polymers that may be used in the present invention are polyoxyethylene, polyethyloxazoline and polyethyleneglycol (PEG). The polymeric  
10       lipids enhance the steric stability of the emulsion, and also PEG with a small molecular weight is a well-known fusogenic agent.

The compositions of the present invention may also comprise an osmotic pressure regulator such as glycerol.

The present invention may also comprise low-molecular weight polyethylene  
15       glycol (average molecular weight in the range of 500-1000) and fusogenic peptide, such as HA gp 41, to improve the transfection efficiency.

The present invention may also comprise materials such as glycolipid, lipopeptide, antibody, and ligand for receptors, viral protein to target specific cells or organs.

20       The present invention may also comprise polycations such as protamine sulfate, histone and polylysine to condense DNA.

The present invention may comprise mono- or poly-anions to alter the interaction between lipid carrier and DNA.

The biologically active material which can be used in the present invention  
25       include DNA, ribonucleic acid (RNA), antisense nucleic acid, ribosome, polynucleotide, oligonucleotide, or other pharmaceutical drugs.

In the case the biologically active material to be deliver is a nucleic acid such as DNA, a carrier must have a positive charge to form a complex with the nucleic acid and also must be within the appropriate size range.

30       The positively charged lipid emulsion or solid lipid nanoparticle of the present invention may be loaded with lipophilic or amphiphilic drugs.  
The lipid emulsion or solid lipid nanoparticle of the present invention may additionally include a hydrophilic drug in the aqueous phase.

For an *in vivo* application, the serum effect must also be considered. The

lipid emulsion of the present invention is physically stable and has high transfection efficiency in the presence of serum.

The lipid emulsion that can deliver biologically active materials such as DNA according to the present invention is prepared by mixing a) 2-30 % of non-  
5 triglycerides oil and b) an aqueous phase comprising 0.01-20 % of one or more emulsifiers including a cationic surfactant and additionally 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety.

The solid lipid nanoparticle that can deliver biologically active materials such as DNA according to the present invention is prepared by mixing a) 2-30 % of  
10 triglycerides having 10 -18 carbons in the hydrophobic tail or ethyl stearate which is heated above its melting temperature and b) a heated aqueous phase comprising 0.01-20 % of one or more emulsifiers including a cationic surfactant and additionally 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety. Upon mixing, the temperature of the aqueous and oil phases must be  
15 maintained above the melting temperature of the oil phase.

The present invention also provides a method of preparing drug-loaded lipid emulsion for the *in vivo* delivery of lipophilic or amphiphilic drugs.

The drug loaded lipid emulsion of the present invention is prepared by mixing a) an oil phase comprising 2-30 % of non-triglycerides oil and 0.1-10 % of lipophilic  
20 or amphiphilic drug and b) an aqueous phase comprising 0.01-20 % of one or more emulsifiers and additionally 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety.

The present invention also provides a method of preparing drug-loaded solid lipid nanoparticle for the *in vivo* delivery of lipophilic or amphiphilic drugs.

25 The drug loaded solid lipid nanoparticle of the present invention prepared by mixing a) an oil phase comprising 2-30 % of triglycerides oil having 10-18 carbons in the hydrophobic tail or ethyl stearate which is heated above its melting temperature and 0.1-10 % of lipophilic or amphiphilic drug and b) an aqueous phase comprising 0.01-20 % of one or more emulsifiers and additionally 0.01-10% of hydrophilic  
30 polymer or phospholipid having a hydrophilic polymeric moiety. Upon mixing, the temperature of the aqueous and oil phases must be maintained above the melting temperature of the oil phase.

It is also possible to prepare the drug-loaded lipid emulsion or solid lipid nanoparticle by solubilizing emulsifiers and lipophilic or amphiphilic drug in the oil

phase completely and subsequently mixing the oil and aqueous phases.

The lipid emulsion that can deliver biologically active materials such as DNA according to the present invention is prepared by mixing a) an oil phase composed of 2-30 % of non-triglycerides oil and 0.01-20 % of one or more emulsifiers including a cationic surfactant and b) an aqueous phase comprising 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety.

The solid lipid nanoparticle that can deliver biologically active materials such as DNA according to the present invention is prepared by mixing a) an oil phase comprising 2-30 % of fat of triglycerides having 10-18 carbons in the hydrophobic tail or ethyl stearate which is heated above its melting temperature, and 0.01-20 % of one or more emulsifiers including a cationic surfactant and b) a heated aqueous phase comprising 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety. Upon mixing, the temperature of the aqueous and oil phases must be maintained above the melting temperature of the oil phase.

The present invention also provides a method of preparing drug-loaded lipid emulsion for the *in vivo* delivery of lipophilic or amphiphilic drugs.

The drug loaded lipid emulsion of the present invention is prepared by mixing a) an oil phase comprising 2-30 % of non-triglycerides oil, 0.01-20 % of one or more emulsifiers and 0.1-10 % of lipophilic or amphiphilic drug and b) an aqueous phase comprising 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety.

The present invention also provides a method of preparing drug-loaded solid lipid nanoparticle for the *in vivo* delivery of lipophilic or amphiphilic drugs.

The drug loaded solid lipid nanoparticle of the present invention is prepared by mixing a) an oil phase comprising 2-30 % of triglycerides oil having 10-18 carbons in the hydrophobic tail or ethyl stearate which is heated above its melting temperature, 0.01-20 % of one or more emulsifiers and 0.1-10 % of lipophilic or amphiphilic drug and b) an aqueous phase comprising 0.01-10% of hydrophilic polymer or phospholipid having a hydrophilic polymeric moiety. Upon mixing, the temperature of the aqueous and oil phases must be maintained above the melting temperature of the oil phase.

The aqueous phase is prepared by preparing liposome according to the method well known in the field. The aqueous and oil phases are mixed by using the method well known in the field. The prepared aqueous and oil phases are heated to



solubilize the components and mixed by using tools such as a homogenizer, sonicator or microfluidizer.

The present invention also is related to a method of transferring biologically active materials such as DNA into cells by using lipid emulsions made of non-  
5 triglyceride and solid lipid nanoparticles made of saturated triglycerides or ethyl stearate.

The present invention also relates to a method of in vivo delivery of lipophilic or amphiphilic drugs by using lipid emulsions made of non-triglyceride and solid lipid nanoparticles made of saturated triglycerides or ethyl stearate.

10 The lipid emulsion and solid lipid nanoparticle according to the present invention deliver the biologically active materials into cells wherein the cell is selected from the group consisting of white blood cells, fibroblasts, cancer cells, cells infected with virus, epithelial cells, endothelial cells, muscle cells, liver cells, endocrine cells, neural cells, dermal cells, germ cells, oocytes, sperms,  
15 hematopoietic cells, fetal cells, M cells, Langerhans islet cells, macrophages, plant cells, animal cells, and immortalized cell lines.

The method of delivering the biologically materials into the target cells according to the present invention is to form a complex between the lipid emulsion or solid lipid nanoparticle with biologically active material.

20 When the lipid emulsion of the present invention is used as the carrier of genetic materials, it can be administered intravenously, intramuscularly, intranasally, intratracheally, subcutaneously, parenterally, by a topical administration, or direct administration to a specific organ.

The lipophilic and/or amphiphilic drug that may be loaded in the lipid emulsion  
25 or the solid lipid nanoparticle includes antivirals, steroidal anti-inflammatory drugs (SAID), non-steroidal anti-inflammatory drugs (NSAID), antibiotics, antifungals, vitamins, hormones, retinoic acid, prostaglandins, prostacyclins, anticancer drugs, antimetabolitic drugs, mitotics, cholinergics, adrenergic antagonists, anticonvulsants, antianxiety agents, major tranquilizers, antidepressants, anesthetics, analgesics,  
30 anabolic steroids, estrogens, progesterones, glycosaminoglycans, polynucleotides, immunosuppressants and immunostimulants.

The invention will be further illustrated by the following examples, but is not limited to the examples given.

Example 1 Preparation of lipid emulsion using egg phosphatidylcholine as an emulsifier

After mixing egg phosphatidylcholine (eggPC) with water at a concentration of 12 mg/ml and incubating for at least 10 minutes for hydration, the mixture was  
5 sonicated using a probe type sonicator (High intensity ultrasonic processor, 600-Watt) for 2 minutes to prepare a liposome solution. Lipid emulsions were prepared with 10 %(v/v) different oils as listed in and the liposome solution by sonication for 2 minutes for 3 times (6 minutes total). The size of the emulsion particles were measured by using Malvern Zetasizer(Malvern Instruments Limited, England) after  
10 diluting the emulsion by 300 times in deionized distilled water. The size of the emulsion particles one day and 20 days after preparation is presented in Table 1 to show the size change of the emulsion droplets with time. The values represent an average from 3 measurements with a single sample. As can be seen in the Table, squalene, a non-triglyceride lipid forms more stable emulsion with a smaller average  
15 droplet size than other emulsions listed. The emulsions so prepared were stored at 4 °C until further experiments.

20

25

30

Table 1

Oil	Size(nm) one day after preparation	Size (nm) 20 days after preparation
Castor oil	246.3	290.2
Coconut oil	246.3	278.2
Corn oil	261.0	298.7
Cottonseed oil	263.0	402.8
Borage oil	247.1	418.0
Fish oil	247.0	271.3
Joboba bean oil	224.0	236.8
Lard oil	282.7	307.9
Linseed oil	354.9	-*
Olive oil	263.0	306.1
Peanut oil	256.8	341.3
Safflower seed oil	283.6	275.9
Sesame oil	263.3	327.8
Soybean oil	249.7	272.0
Squalene	191.7	216.2

\* The particle size increased beyond measurable values.

#### Example 2. Preparation of cationic lipid emulsions using oils having different o/w interfacial tensions

Lipid emulsions were prepared using various oils (10 %(v/v)) and DOTAP as an emulsifier. DOTAP was mixed with water at a concentration of 24 mg/ml to solubilize the lipids at 37 °C. The lipid solution was sonicated by using a probe type sonicator for 2 minutes to form a liposome solution. Various oils (10 %(v/v)) were added to the liposome solutions and sonicated for 2 minutes 3 times (total 6 minutes) to form lipid emulsions. The size and the zeta potential of the lipid emulsions were measured by using Malvern Zetasizer (n=3). The results are listed in Table 2. The lipid emulsion made of oils having higher o/w interfacial tension has a smaller average droplet sizes than those made of oils having lower o/w interfacial tension. The emulsion particle sizes were below 200 nm with an exception of linseed oil emulsion. The zeta-potential of the emulsions were  $48.4 \pm 8.5$  mV (n=3). The emulsions were kept at 4 °C until further experiments.

Table 2

	Oil	Size (nm)	Zeta-potential (mV)
Liposome		108.5 $\pm$ 45.1	48.4 $\pm$ 8.5
Emulsion A	Linseed oil	220.4 $\pm$ 23.1	50.1 $\pm$ 5.4
Emulsion B	Soybean oil	204.1 $\pm$ 18.4	57.7 $\pm$ 6.7
Emulsion C	Squalane	168.4 $\pm$ 10.4	
Emulsion D	Squalene	157.5 $\pm$ 8.9	64.5 $\pm$ 7.2

Example 3. Stability of lipid emulsions made of various oils in the presence of serum

5 The stability of the DOTAP liposomes and emulsions were compared by measuring the emulsion particle sizes in the presence of serum. The result of the measurements was listed in Table 3. Even with 0.5 % of serum, the sizes of the liposomes increased by 2.7 times. In the case of lipid emulsions, there are no apparent changes in the sizes in the presence of serum. One of the problems in  
 10 delivering genes using liposomes was the formation of large insoluble aggregates that can attenuate the transfection efficiency. The lipid emulsions of the present invention overcame such a problem by maintaining the small size with narrow size distribution in the presence of serum.

15 Table 3. Stability of lipid gene carriers in the presence of serum

	Oil	Size (nm)	
		no serum	0.5 % serum
Liposome		108.5 $\pm$ 45.1	295.3 $\pm$ 85.9
Emulsion A	Linseed oil	220.4 $\pm$ 23.1	261.5 $\pm$ 12.4
Emulsion B	Soybean oil	204.1 $\pm$ 18.4	201.3 $\pm$ 11.1
Emulsion D	Squalene	157.5 $\pm$ 8.9	174.0 $\pm$ 5.5

Example 4. Preparation of cationic solid lipid nanoparticles

Lipid emulsions were prepared using various oils (10 %(v/v)) and DOTAP as an emulsifier. DOTAP was mixed with water at a concentration of 24 mg/ml to  
 20 solubilize the lipids at 37 °C. The lipid solution was sonicated by using a probe type

sonicator for 2 minutes to form a liposome solution. Trilaurin or ethyl stearate according to Table 1 were heated to 50 °C. Melted oils (10 %(v/v)) were added to the liposome solutions and sonicated for 2 minutes for 3 times (total 6 minutes) to form lipid emulsions at 50 °C. The lipid emulsion was slowly cooled at room temperature to solidify melted oils and as a resultant to form solid lipid nanoparticles. The sizes and the zeta potential of the lipid emulsions were measured by using Malvern Zetasizer (n=3). The results are listed in Table 4. The emulsions were kept at 4 °C until further experiments.

10 Table 4

Fat	Size(nm)
Trilaurin	181.2 ± 13.2
Ethyl stearate	183.2 ± 1.4

#### Example 5. Cell culture and isolation of plasmid DNA

##### Cell Culture

COS-1 cells (kidney, SV40 transformed, African green monkey) were grown in 5 % CO<sub>2</sub> incubator in DMEM supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin.

##### Isolation of plasmid DNA

A plasmid pCMV-CAT encoding a chloramphenicol acetyl transferase driven by a human cytomegalovirus immediate-early promoter was purchased from Invitrogen (Groningen, Netherlands). The pCMV-beta encoding a  $\beta$ -galactosidase driven by a human cytomegalovirus immediate-early promoter was purchased from Clontech, Inc.

The plasmids were amplified in a *E. coli* DH5- $\alpha$  strain and purified by using a Quiagen mega-kit (Quiagen Inc., Chatsworth, CA) according to the manufacturer's instruction. DNA purity was determined by agarose gel electrophoresis and by measuring optical density. DNA having O.D.<sub>260</sub>/O.D.<sub>280</sub>  $\geq 1.8$  were used in this study.

#### Example 6: Complex formation between cationic lipid emulsion and DNA

The complex formation between DNA and the lipid emulsion prepared in

Example 2 was observed by using a gel electrophoresis technique. Lipofectamine and DOTAP liposomes were used as controls. DNA (1  $\mu$ g) was mixed with appropriate amounts of lipid carriers in a test tube, and the complexes were incubated for 30 minutes at room temperature. The complexes were loaded on a 1 % agarous gel containing ethidium bromide and electrophoresed in Tris-acetate-EDTA buffer solution at pH 8.0. The gel was visualized under a UV light as shown in Figure 4.

As shown in Figure 4, 2  $\mu$ l of Lipofectamine formed a complex with 1  $\mu$ g of DNA. Likewise the emulsions also formed emulsion/DNA complexes effectively.

10

#### Example 7: Protection of DNA against PLAA

DNA complexes were formed with the Lipofectamine, DOTAP liposomes and cationic lipid emulsions as in example 6. Different concentrations of poly-L-aspartic acid (PLAA) were added to the complex. Since PLAA is an anionic polymer, it can dissociate the anionic DNA from the complex. Therefore, the dissociation of DNA from the complex in the presence of PLAA is an indicator of the strength of the complex. DNA was dissociated from the Lipofectamine/DNA and DOTAP liposome/DNA complexes at or above the equivalency between PLAA/DNA of 1.25. On the other hand, the BRC 001-DNA complex remained stable in at the equivalence of 640. The result indicates that the lipid emulsion/DNA complex is stronger than liposome/DNA complexes in the presence of an anionic competitor. Therefore, it is expected that the emulsion carrier can exhibit superior transfection efficiency in the presence of biological barriers including serum or mucus layer.

#### Example 8: Gene transfection efficiency in COS-1 cell line by cationic lipid emulsions

COS-1 cells were seeded at  $1 \times 10^4$  cells in a 96-well plate ca. 12 hours prior to transfection. pCMV-beta plasmid DNA (0.5  $\mu$ g) were mixed with DOTAP liposome and lipid emulsions as example 2 at amounts to contain 2  $\mu$ g of DOTAP. The mixtures were diluted in 2  $\mu$ l of serum free DMEM medium. The mixtures were completely shaken 10 times by inversion during 30 min incubation period. After cleaning the COS-1 cells with serum free RPMI 1640, 160  $\mu$ l serum free media and carrier-DNA mixture were added to cells. After a 1 h incubation at 37 °C in a 5 % carbon dioxide incubator, the cells were washed with serum free DMEM to remove

the remaining carrier-DNA complex in the solution. The cells were incubated in DMEM containing 10% serum for 24 hours. The transfected cells were harvested after the incubation. Cells were washed with 200  $\mu$ l PBS, harvested with 50  $\mu$ l of lysis solution (0.1% Triton X-100, 250 mM Tris, pH8.0), and lysed by means of a freeze-thaw cycle. In each well, 50  $\mu$ l of PBS contained 0.5 % of bovine serum albumin. The substrate solution (150  $\mu$ l of 1mg/ml chloramphenicol red galactopyranoside) was added at room temperature for an hour for enzymatic reaction. A recombinant  $\beta$ -galactosidase was used as a standard. The optical density was measured at 570 nm (n=3). The transfection efficiency is expressed as the relative  $\beta$ -galactosidase activity as shown in Table 5.

Table 5. Transfection efficiency and cytotoxicity using lipid emulsions having different oils.

	oil	Transfection efficiency (mU/well)		Cell proliferation rate (%)
		no serum	80 % serum	
Liposome		0.73 $\pm$ 0.13	0.04 $\pm$ 0.01	78.4 $\pm$ 13.5
Emulsion A	Linseed oil	0.22 $\pm$ 0.10	0.08 $\pm$ 0.02	77.1 $\pm$ 12.4
Emulsion B	Soybean oil	0.21 $\pm$ 0.07	0.12 $\pm$ 0.02	85.7 $\pm$ 11.7
Emulsion C	Squalane	0.30 $\pm$ 0.08	0.18 $\pm$ 0.08	85.9 $\pm$ 12.2
Emulsion DI	Squalene	0.37 $\pm$ 0.09	0.22 $\pm$ 0.07	89.5 $\pm$ 10.2

#### Example 9. Effect of serum on transfection efficiency in COS-1 cell line

To test the serum effect on DNA transfection efficiency, carrier-DNA complexes were prepared in DMEM without serum. The cells were washed with DMEM without serum. Transfection was performed in the presence of 80 % serum (in 160  $\mu$ l of DMEM) according to the method in Example 8. The transfection efficiency in the presence of 80 % serum is listed in Table 5.

#### Example 10. Cytotoxicity

Live cells is quantified by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) assay. A tetrazolium ring opening reaction can take place only in the presence of the live cells with active mitochondria. This

yellow-to-purple reaction is quantified by using the scanning multiwell spectrophotometer (ELISA reader).

The COS1 cells were harvested from exponential phase culture by trypsinization, counted and plated in 96-well microplates. A concentration of  $1 \times 10^4$  cells/well was found suitable. Plates were incubated at 37°C for 1 day under a humidified atmosphere. The lipid emulsions were diluted in growth medium to a final volume of 200  $\mu$ l and added to the cells and incubated for 24 hours. After exposure with a lipid emulsion, the cells were washed twice with PBS. MTT was dissolved in PBS, filter-sterilized and stored at 4°C until further experiment. Two hundred (200)  $\mu$ l of fresh media and 50  $\mu$ l of 0.5 % MTT solution were added to each well and incubated for 4 hours at 37°C to allow intracellular metabolism. Formazan crystals were dissolved by adding 200  $\mu$ l of dimethyl sulfoxide (DMSO). Sorensen's glycine buffer (25  $\mu$ l) was added. The absorbance was measured at 570 nm on an Elisa reader [SOFTmax PRO (Molecular Devices corporation, California, U.S.A.)] as shown in Table 5.

The transfection efficiency is lower for the lipid emulsions than for the liposomes without serum. However, the transfection efficiency of the liposome decreased by 18.2 times upon adding serum. In contrast, the transfection efficiency of the lipid emulsion decreased on by 1.6-2.7 times upon adding serum. Among the lipid emulsions, the transfection efficiency of the squalene emulsion was better than other emulsion carriers with or without serum. The squalene emulsion has a superior physical stability with or without serum. This physical stability may provide an ability to deliver genes to cells in the presence of many destabilizing factors as *in vivo* applications. Moreover, the cytotoxicity of the emulsions was lower than liposomes. The squalene emulsion had a lowest cytotoxicity having more than 89 % of cell proliferation rate.

#### Example 11. Complex formation between cationic solid lipid nanoparticle and DNA

The cationic solid lipid nanoparticle made of trilaurin in Example 4 and DNA were mixed at different ratios in a buffer solution and negatively stained with 1 % uranyl acetate to observe the complex under transmission electron microscope at a magnification ratio of 50,000. Figure 6A is pure DNA. Figures 6C and 6D represents the solid lipid nanoparticle/DNA complexes.



Example 12. Transfection with cationic solid lipid nanoparticle

Cationic solid lipid nanoparticle prepared with trilaurin was used for the in vitro transfection. The  $\beta$ -galactosidase activity was  $0.24 \pm 0.07$  and  $0.11 \pm 0.02$  mU/well without or with 80 % serum, respectively. The cell proliferation rate was  $82.6 \pm 15.6$  % indicating low cytotoxicity of the solid lipid nanoparticles. The results indicate that the solid lipid nanoparticles can be used as a gene carrier.

Example 13. Increase in the transfection efficiency by adding DOPE as a helper lipid to the cationic lipid emulsion.

Squalene emulsions were prepared by using an additional emulsifier, DOPE to increase transfection efficiency. The helper lipid, DOPE has a fusogenicity at room temperature and is known to disturb endosomal membrane to increase the transfection efficiency.

The liposome and squalene emulsion were prepared as in Example 2, except the emulsifier was 24 mg/ml DOTAP/DOPE mixtures instead of pure DOTAP. The weight ratio between DOTAP and DOPE were 11:1, 7:1, 5:1, 3:1, 5:3, 1:1, 1:3 and 1:5. Transfection was performed by following the method in Example 8. Also, the serum effect was studied using the method in Example 9. The results are illustrated in Figure 7. The transfection efficiency was maximum at DOTAP/DOPE ratio of 5:1 for liposome and lipid emulsion. At a higher DOPE concentration, the transfection efficiency decreases probably due to the instability of the lipid carriers and the complexes resulted by the addition of the DOPE.

Example 14. Increase in the transfection efficiency by adding diolein as a helper lipid to the cationic lipid emulsion.

Squalene emulsions were prepared by using an additional emulsifier, diolein, to increase transfection efficiency. The present inventors have tested the possibility of using diolein, which has not been used as a helper lipid, to increase the transfection activity.

The liposome and squalene emulsion were prepared as in Example 2, except the emulsifier was DOTAP/diolein mixture (9:1 by weight) instead of pure DOTAP. Transfection was performed by following the method in Example 8. Also, the serum effect was studied using the method in Example 9. The results are illustrated in Table 6.

The transfection efficiency was increased by adding diolein for liposome and lipid emulsion. Interestingly, cytotoxicity of the liposome decreased upon adding diolein. This result is in contrast with the increase in cytotoxicity of the liposome upon adding DOPE. The decrease in the cytotoxicity may come from the fact that diolein activates protein kinase C which regulates cell function. Therefore, unlike DOPE, diolein may be a better helper lipid that increases the transfection efficiency with a lowered cytotoxicity.

Table 6. Influence of diolein on Transfection efficiency and cell proliferation rate.

	Oil	Emulsifier	Size (nm)	Transfection efficiency (mU/well)		Cell proliferation rate (%)
				no serum	80% serum	
Liposome	-	DOTAP	148.5 $\pm$ 44.1	0.73 $\pm$ 0.13	0.04 $\pm$ 0.01	78.4 $\pm$ 13.5
	-	DOTAP/ DIOLEIN	185.2 $\pm$ 21.1	0.98 $\pm$ 0.12	0.07 $\pm$ 0.04	83.4 $\pm$ 14.8
Lipid emulsion	Squalene	DOTAP	145.1 $\pm$ 18.4	0.35 $\pm$ 0.09	0.20 $\pm$ 0.04	82.7 $\pm$ 10.4
	Squalene	DOTAP/ DIOLEIN	165.2 $\pm$ 19.2	0.46 $\pm$ 0.07	0.22 $\pm$ 0.07	85.6 $\pm$ 11.1

Example 15. Increase in the transfection efficiency by adding DOPE and diolein simultaneously as helper lipids to the cationic lipid emulsion.

Both DOPE and diolein in Examples 13 and 14, respectively, were used as helper lipids to DOTAP in squalene emulsions in transfection. The weight ratio between DOTAP and DOPE was set at 5:1 and the amount of diolein was changed. The squalene emulsion was prepared as in Example 2, except the emulsifiers were DOTAP, DOPE and diolein mixtures instead of pure DOTAP. The weight ratio between (DOTAP+DOPE) and diolein were 1:0, 7:1, 5:1, 3:1, 1:1 and 1:3. Transfection was performed by following the method in Example 8. Also, the serum effect was studied using the method in Example 9. The results are illustrated in Figure 9.

The transfection efficiency was maximum at (DOTAP+DOPE)/DOPE ratio of 5:1. By adding DOPE and diolein, DOPE and diolein may have provided fusogenicity and diolein may have acted as a protein kinase C activator.

Transfection activity, however, decreased at higher concentrations indicating that diolein changes the stability of emulsion/DNA complex.

Example 16. Transfection efficiency and cytotoxicity by adding an emulsifier with polyethylene glycol moiety.

Emulsifier with polyethylene glycol (PEG) moiety may increase the stability of emulsions by providing a steric hindrance. Also, low molecular weight PEG unit may also provide a fusogenicity. Therefore, different PEG-lipids were used as a co-emulsifier to form emulsions. Squalene emulsions were prepared by using 24 mg/ml DOTAP/DOPE (5:1 by weight) and different polymeric emulsifiers. Different amounts (10, 20, 30 and 50 % (w/w) of the weight of DOTAP/DOPE mixture) of polymeric emulsifiers, Tween 80, PEG<sub>2000</sub>PE, HCO 60 and Pluronic F68, were added to form emulsions. Transfection and cytotoxicity studies were performed by following the methods in Examples 8 and 10. Depending on the type of polymeric lipid, optimum transfection efficiency was obtained at different DOTAP concentrations. The results in Table 7 show the optimum efficiency for each emulsion.

In the case of liposomes, the transfection efficiency in the presence of 80 % serum decreased significantly by adding polymeric lipids. In contrast, the transfection efficiency with 80 % serum was at least 50 % of that without serum when lipid emulsions were used as gene carriers. The transfection efficiency increased for liposome and emulsion upon adding Tween 80. However, other polymeric lipids lowered the transfection efficiency due to the steric hindrance of the polymeric moiety. As a result, larger amounts of the carrier were needed for the interaction between cationic lipid and DNA with an increased cytotoxicity. Therefore, Tween 80 was the lipid of choice that provides emulsion stability without hindering the interaction between emulsion and DNA.

Table 7.

Formulation	Non-ionic surfactant	DOTAP (mg)*	Size (nm)	Transfection efficiency (mU/well)		Cell proliferation rate (%)
				No serum	80% serum	
Liposome A	-	2	154.4 $\pm$ 25.1	0.90 $\pm$ 0.31	0.08 $\pm$ 0.01	78.9 $\pm$ 18.5
Liposome B	Tween80	3	145.6 $\pm$ 12.1	1.51 $\pm$ 0.28	0.12 $\pm$ 0.05	76.0 $\pm$ 11.3
Liposome C	PEG <sub>2000</sub> PE	5	151.41 $\pm$ 19.4	0.54 $\pm$ 0.12	0.04 $\pm$ 0.02	82.0 $\pm$ 12.3
Liposome D	HCO60	5	140.2 $\pm$ 0.98	0.50 $\pm$ 0.10	0.06 $\pm$ 0.02	85.0 $\pm$ 10.7
Emulsion A	-	2	200.1 $\pm$ 08.7	0.62 $\pm$ 0.15	0.38 $\pm$ 0.11	89.3 $\pm$ 15.4
Emulsion B	Tween80	4	186.3 $\pm$ 13.2	1.05 $\pm$ 0.18	0.52 $\pm$ 0.13	79.8 $\pm$ 18.2
Emulsion C	PEG <sub>2000</sub> PE	8	190.2 $\pm$ 10.4	0.41 $\pm$ 0.10	0.23 $\pm$ 0.09	81.8 $\pm$ 14.5
Emulsion D	HCO60	8	162.0 $\pm$ 08.9	0.33 $\pm$ 0.11	0.24 $\pm$ 0.07	83.2 $\pm$ 12.3
Emulsion E	F68	10	232.2 $\pm$ 12.3	0.20 $\pm$ 0.09	0.10 $\pm$ 0.06	78.3 $\pm$ 15.7

\* Amount of DOTAP required to form a complex with 0.5 g DNA that shows maximum transfection efficiency.

#### Example 17. Increase in the transfection efficiency by adding Tween 80

- 5 Squalene emulsions were prepared by using 24 mg/ml DOTAP/DOPE (5:1 by weight) and different polymeric Tween 80. The amount of Tween 80 was 0, 5, 10, 15 and 20 %(w/w) in addition to DOTAP/DOPE. Transfection and was performed by following the method in Example 8. The results are shown in Figure 9. The transfection efficiency increased as the amount of Tween 80 increased up to 10 %.
- 10 At higher than 10 % of Tween 80, however, the efficiency decreased probably due to an increase of the steric hindrance of the polyethylene glycol moiety.

#### Example 18. Increase in the emulsion stability by adding Tween 80

- To investigate whether Tween 80 increases the emulsion stability, squalene
- 15 emulsions having 24 mg/ml DOTAP/DOPE (5:1 by weight) with or without 10 %(w/w) Tween 80 as in Example 17 were prepared. Emulsions were diluted by 300 fold in phosphate buffered saline with 0.5 % serum and 0.1 % sodium azide to measure absorbance at 600 nm (n=3). The absorbance immediately after dilution was set to 100 % as shown in Figure 10. Unlike the emulsion made without Tween 80, the

absorbance of the emulsion with Tween 80 did not change with time indicating that the emulsion stability increased by adding Tween 80.

#### Example 19. Preparation methods

Lipid emulsion having 10 %(w/w) Tween 80 of Example 17 was prepared by two different preparation methods; microfluidization and sonication. Size and transfection efficiency were compared. Preparation method of the sonicated emulsion was same as Example 2. The method using microfluidization was as follows. Oil and aqueous phases were heated to 70 °C to solubilize the components completely before mixing the two phases. The emulsion was prepared by mixing the two solutions with a high-speed homogenizer (T-25-Ultra-Turrax, S25-18G, IKA Werke, Janke & Kunkel GmbH & Co KG, Germany) at 8000 rpm for 10 min. The mixture was passed 10 times through a Microfluidizer (Microfluidics Co., Newton, MA) with an exit air pressure of 80 psi. The emulsion so prepared was stored at 4 °C until further use. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1. Transfection was performed as in Example 8. The results are listed in Table 8.

Table 8. Characteristics of lipid emulsions made by different preparation methods.

Method of preparation	Size (nm)	Transfection efficiency (mU/well)	
		No serum	80% serum
Microfluidization	178.5 ± 12.7	1.10 ± 0.20	0.54 ± 0.14
Sonication	186.3 ± 13.2	1.05 ± 0.18	0.52 ± 0.13

Emulsions made by two different methods produced similar results (n=3). It is desirable to use microfluidization method for mass-production and to use sonication for small-scale preparation.

#### Example 20. Effect of pre-treatment with protamine sulfate on the size of the complex

Protamine sulfate is a polycationic protein and forms a protamine sulfate/DNA complex by interacting with and condensing anionic DNA. Also protamine sulfate has a nucleus targetting moiety that can promote a transfer of the complex to

nucleus to increase the transfection activity.

In forming a complex between emulsion and DNA, protamine sulfate was mixed with DNA before adding an emulsion solution.

Protamine sulfate, at amounts of 0.5, 1.0 and 1.5  $\mu\text{g}/\text{well}$ , were added to DNA 5 15 min before adding the emulsion. Half an amount of the emulsion used in Example 6 (2  $\mu\text{g}$  of DOTAP in the emulsion) was added to the protamine sulfate/DNA mixture. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1. Table 9 shows that the size of the complex does not increase by adding protamine sulfate. Addition of 10 protamine sulfate may lower the amount of cationic lipid emulsion and as a result may lower the emulsion related cytotoxicity.

Table 9.

Formulation n	emulsifier	DNA	Protamine sulfate	Size (nm)
Liposome	DOTAP/DOPE	-	-	108.5 $\pm$ 15.1
	DOTAP/DOPE	+	-	174.2 $\pm$ 08.9
	<b>DOTAP/DOPE</b>	<b>+</b>	<b>+</b>	<b>100.2 <math>\pm</math> 06.9</b>
	DOTAP/DOPE/Tween80	-	-	120.4 $\pm$ 13.1
	DOTAP/DOPE/Tween80	+	-	138.4 $\pm$ 10.1
	<b>DOTAP/DOPE/Tween80</b>	<b>+</b>	<b>+</b>	<b>112.2 <math>\pm</math> 04.9</b>
Lipid emulsion	DOTAP/DOPE	-	-	121.1 $\pm$ 05.1
	DOTAP/DOPE	+	-	179.4 $\pm$ 05.9
	<b>DOTAP/DOPE</b>	<b>+</b>	<b>+</b>	<b>119.2 <math>\pm</math> 03.9</b>
	DOTAP/DOPE/Tween80	-	-	130.9 $\pm$ 04.9
	DOTAP/DOPE/Tween80	+	-	163.5 $\pm$ 05.5
	<b>DOTAP/DOPE/Tween80</b>	<b>+</b>	<b>+</b>	<b>125.4 <math>\pm</math> 03.9</b>

Example 21. Increase in transfection efficiency by adding protamine sulfate.

Emulsion/DNA complex was made after pre-treatment with protamine sulfate as in Example 20, and transfection was performed as in Example 8. Transfection efficiency in the presence of serum was also determined by following the method in  
5 Example 9. The results are shown in Figure 11. Transfection efficiency by using emulsion or protamine sulfate alone was 7-10 times lower than that by using emulsion/DNA complex pre-treated with DNA. The highest transfection efficiency was obtained with 1.0  $\mu$ g protamine sulfate.

Interestingly, pre-treatment with protamine sulfate may not increase the  
10 transfection efficiency when liposome was used instead of emulsion. Moreover, transfection efficiency was maintained in the presence of serum when lipid emulsion was used as a gene carrier.

Example 22. Transfection efficiency in different cell lines

15 Lipid emulsion containing DOTAP/DOPE/Tween80 as in Example 19 was used as a transfection reagent in different cell lines using the method in Example 8 except that CV-1 or NIH3T3 cell lines was used instead of COS-1. Figure 12 shows that the lipid emulsion may be used as gene carriers for these cell lines.

20 Example 23. Preparation of lipid emulsion loaded with rifampicin

Oil phase comprising 1g of oils in Table 10, 100 mg egg PC and 80 mg PEG<sub>2000</sub>PE were heated to 55 °C to solubilize the components completely. Rifampicin (10 mg) was added to the oil phase. The emulsion was prepared by adding 10 ml PBS and by sonicating for 2 min. The size of the emulsion particles was  
25 measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1 (Table 10)

Table 10.

Oil	Size (nm)
Linseed oil	226.5 $\pm$ 3.12
Soybean oil	218.1 $\pm$ 2.83
Squalene	224.0 $\pm$ 3.28

Example 24. In vitro release of rifampicin from lipid emulsions

Three milliliters of emulsions prepared in Example 23 were put in dialysis bags, each end tightened by closures. The emulsion-filled bags were sunk in 10 ml PBS contained in 50 ml conical tubes. The conical tubes were put in shaking water bath at 37 °C. As a comparative example, release rate of rifampicin in PBS was compared. Concentration of rifampicin was measured by fluorescence spectroscopy.

As shown in Figure 13, the release rate was slowest from squalene emulsion by showing 0<sup>th</sup> order release pattern. Release rate was fastest for the rifampicin solution followed by linseed oil emulsion and by soybean oil emulsion.

Example 25. Preparation of lipid emulsion loaded with diclofenac sodium

Emulsions were prepared as in Example 23 except that 3 mg diclofenac sodium was loaded instead of rifampicin. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1

Table 11.

Oil	Size (nm)
Linseed oil	210.0 ± 0.61
Soybean oil	222.5 ± 2.39
Squalene	235.8 ± 0.36

Example 26. In vitro release of diclofenac sodium from lipid emulsions

*In vitro* release of diclofenac sodium from the emulsions prepared in Example 25 was performed by following the method in Example 26. As a comparative example, release rate of diclofenac sodium in PBS was compared. Concentration of diclofenac sodium was measured by HPLC. Release of diclofenac was faster than that of rifampicin since diclofenac was more hydrophilic.

As shown in Figure 14, the release rate was slowest from squalene emulsion than other emulsions. However, the difference in the release rate is much less pronounced than in case of rifampicin release. Also, release of diclofenac solution was the fastest of all.



Example 27. Estimation of lethal dose

Squalene emulsion prepared in Example 2 was used to estimate the lethal dose. 200 microliters of emulsions diluted by 1/2, 1/4, 1/8 and 1/16 were injected intravenously to Balb/C mice (groups of 6 mice) to observe the survival rate after 24 hours. The results are shown in Table 12. The dose where half of the mice in the group die (LD<sub>50</sub>) is approximately 1.6 g DOTAP/kg.

Table 12.

Dilution ratio	DOTAP (mg)	Squalene (mg)	Percent survival
1/2	2.4	8	402
1/4	1.2	4	16.6
1/8	0.6	2	66.6
1/16	0.3	1	100

10 Example 28. Systemic gene delivery by intravenous administration of emulsion/DNA complex (*In vivo* experiment)

Emulsion/DNA complexes as well as liposome/DNA complexes were delivered systemically by intravenous administration. Complexes between 1.7 µl lipid emulsions prepared in Example 2 and 10 µg pCMV-luc + were injected intravenously through the tail vein of 30 g Balb/C mouse. For comparison, liposome/DNA complex and naked DNA were also administered. The mice were sacrificed 22 hours after the injection to analyze the expression of luciferase in each organ. Heart, liver, lung, kidney and spleen were obtained for analysis. Expression of luciferase was measured by following the protocols provided by Promega. Briefly, each organ was homogenized after adding 4 µl of lysis buffer per 1 mg tissue. The ground tissue underwent two freeze-thaw cycles and centrifuged for 2 min at 10,000 g to obtain clear supernatant solution. The supernatant solution was stored at -20 °C until analysis. Ten microliters of the solution was mixed with 100 µl of buffer solution for luciferase analysis to measure relative light unit. As shown in Figure 15, the expression rate in each organ, especially in the lung, was pronounced with squalene emulsion as a gene carrier.

Example 29. Systemic gene delivery by intraveous administration of emulsion/DNA complex (*In vivo* experiment)

In vivo experiments were performed as in Example 28 except that complexes having 10.5  $\mu$ l lipid emulsions and 50  $\mu$ g DNA was used. For comparison, liposome/DNA complexes and naked DNA were also administered. The mice were sacrificed 22 hours after the injection to analyze the expression of luciferase in each organ. Heart, liver, lung, kidney and spleen were obtained for analysis. As shown in Figure 16, the expression rate in each organ, especially in the lung, was pronounced with squalene emulsion as a gene carrier.

10

Example 30. Systemic gene delivery by intraveous administration of emulsion/DNA : Effect of emulsifiers with PEG moiety

In vivo experiments were performed by following the method in Example 28. DNA (10  $\mu$ g) was complexed with lipid emulsions prepared as in Example 18. Amount of emulsions in the complexes was controlled to show maximum transfection efficiency *in vitro* in Example 18. For comparison, liposomes having same lipid composition was complexed with DNA for the intraveous injection. The mice were sacrificed 22 hours after the injection to analyze the expression of luciferase in each organ. Heart, liver, lung, kidney and spleen were obtained for analysis. When DOTAP/DOPE and DOTAP/DOPE/Tween 80 were used as emulsifier systems, emulsions showed higher transfection efficiency than the liposomes used. The DOTAP/DOPE/Tween 80 emulsion, had a high in vivo transfection efficiency especially in the lung.

Example 31. Systemic gene delivery by intraveous administration of emulsion/DNA : Effect of emulsifiers with PEG moiety

*In vivo* experiments were performed by following the method in Example 28. DNA (pCMV-luc+, 10  $\mu$ g) was complexed with 30  $\mu$ g of protamine sulfate and incubated for 15 min at room temperature. Appropriate amount of lipid emulsion prepared as in Example 18 was added by following the method in Example 21 to form a complex. Amount of emulsions in the complex was controlled to show maximum transfection efficiency *in vitro* in Example 18. For comparison, liposomes having same lipid composition was complexed with DNA for the intraveous injection. The mice were sacrificed 22 hours after the injection to analyze the expression of

luciferase in each organ. Heart, liver, lung, kidney and spleen were obtained for analysis. When DOTAP/DOPE and DOTAP/DOPE/Tween 80 were used as emulsifier systems, emulsions showed higher transfection efficiency than the liposomes used. The DOTAP/DOPE/Tween 80 emulsion, had a high in vivo  
5 transfection efficiency especially in the lung.

Example 32. Gene delivery by nasal instillation of emulsion/DNA

Emulsion/DNA complexes in Examples 2, 13 and 16 were administered by nasal administration. Complex between DNA (pCMV-luc+, 20 µg) and emulsion was  
10 administered to Balb/c Mice (30 g). For comparison, liposome/DNA complex as well as naked DNA were administered. The mice were sacrificed 20 hours after the injection to analyze the expression of luciferase in the nasal cavity and lung. Expression of luciferase was measured by following the protocols provided by Promega. As shown in Figure 19, squalene emulsions showed prominently higher  
15 luciferase expression rate than liposome or naked DNA. Interestingly, squalene emulsion made with DOTAP/DOPE had higher luciferase expression in nasal cavity whereas DOTAP/DOPE/Tween 80 was more efficient in the lung.

Example 33. Preparation of lipid emulsion loaded with diclofenamic acid

20 Emulsions were prepared as in Example 23 except that 3 mg diclofenamic acid was loaded instead of rifampicin. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1.

Table 13

Oil	Size (nm)
Linseed oil	213.2 ± 3.03
Soybean oil	240.1 ± 1.14
Squalene	239.0 ± 1.23

25

Example 34. In vitro release of diclofenac sodium from lipid emulsions

*In vitro* release of diclofenac sodium from the emulsions prepared in Example 32 was performed by following the method in Example 24. As a comparative example, release rate of diclofenac sodium in PBS was compared. Concentration

of diclofenac sodium was measured by HPLC.

Diclofenamic acid are more hydrophobic than its salt form. As shown in Figure 20, the release rate was slowest from squalene emulsion than other emulsions.

5 Example 35. Preparation of solid lipid nanoparticles loaded with cyclosporin

Oil phase comprising 1g of ethyl stearate, 100 mg egg PC and 80 mg PEG<sub>2000</sub>PE was heated to 55 °C to solubilize the components completely. Cyclosporin (20 mg) was added to the oil phase at 55 °C. The solid lipid nanoparticles were prepared by adding 10 ml PBS and by sonicating for 2 min. The size of the emulsion particles was measured by using Malvern Zetasizer after diluting the emulsion by 300 times in water as in Example 1 (Table 10). Also, emulsions were prepared as in Example 23 except that 20 mg cyclosporin was loaded instead of rifampicin.

15 Table 14.

Formulation	Size (nm)
Linseed oil emulsion	199.6 ± 2.6
Soybean oil emulsion	200.5 ± 0.7
Ethyl stearate SLN	180.1 ± 0.7

Example 36. Freeze-drying of solid lipid nanoparticles loaded with cyclosporin

The solid lipid nanoparticles loaded with cyclosporin were freeze dried for convenient storage. Solid lipid nanoparticles resuspended in water had an average particle size of ca. 500 nm.

**Claims;**

1. A lipid emulsion comprising: 2-30 % of non-triglyceride oil; 0.01-20 % of one or more emulsifiers including a cationic surfactant; and, water to 100 %.
2. A solid lipid nanoparticle comprising: 2-30 % of fat of triglycerides having 10-18 carbons in each hydrophobic tail or ethyl stearate; 0.01-20 % of one or more emulsifiers including a cationic surfactant; and, water to 100 %.
3. A lipid emulsion loaded with a drug comprising: 2-30 % of squalene or squalane; 0.01-20 % of one or more emulsifiers; 0.1-10 % lipophilic or amphiphilic drug; and, water to 100 %.
4. A solid lipid nanoparticle loaded with a drug comprising: 2-30 % of ethyl stearate; 0.01-20 % of one or more emulsifiers; 0.1-10 % lipophilic or amphiphilic drug; and water to 100 %.
5. A method of preparing a lipid emulsion comprising: a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers including a cationic emulsifier and b) a second step of preparing emulsion of said aqueous phase with 2-30 % of non-triglyceride oil.
6. A method of preparing a solid lipid nanoparticle comprising: a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers including a cationic emulsifier with water; and, b) a second step of mixing said aqueous phase with 2-30 % of fat of triglycerides having 10-18 carbons in each hydrophobic tail or ethyl stearate.
7. A method of preparing a lipid emulsion loaded with a drug comprising: a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers with water, b) a second step of preparing an oil phase by mixing 0.1-10 % of lipophilic or amphiphilic drug and 2-30 % of non-triglyceride oil; and, c) a third step of preparing drug-loaded emulsion by mixing the aqueous and oil phases prepared in the first and second steps, respectively.

8. A method of preparing a solid lipid nanoparticle loaded with a drug comprising: a) a first step of preparing an aqueous phase by mixing 0.01-20 % of one or more emulsifiers; b) a second step of mixing 0.1-10 % of lipophilic or amphiphilic drug and 2-30 % of ethyl stearate; and, c) a third step of preparing the drug-loaded solid lipid nanoparticle by mixing the aqueous and oil phases prepared in first and second steps, respectively.
9. The emulsion according to claim 1, further comprising 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.
10. The emulsion according to claim 1, wherein the non-triglycerides of step a) is squalene or squalane.
11. The emulsion according to any of claims 1, 9 or 10, wherein the emulsifier further comprises a phospholipid or a non-ionic surfactant.
12. The emulsion according to claim 1, wherein the cationic surfactant is selected from the group consisting of, 1,2-dimyristoyl-3-trimethylammonium-propane, 1,2-dipalmitoyl-3-trimethylammonium-propane, 1,2-distearoyl-3-trimethylammonium-propane, 1,2-dioleoyl-3-trimethylammonium-propane, 1,2-dimyristoyl-3-dimethylammonium-propane, 1,2-dipalmitoyl-3-dimethylammonium-propane, 1,2-dilauroyl-3-dimethylammonium-propane, 1,2-distearoyl-3-dimethylammonium-propane, dimethyldioctadecylammonium chloride, N-[1-(1,2-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride, 1,2-dioleoyl-3-ethylphosphocholine, and other cationic phospholipid.
13. The emulsion according to any of claims 1, 9 or 10, further comprising glycerol or fusogenic peptides.
14. The emulsion according to claim 13, wherein the fusogenic peptide is

polyethylene glycol of MW. 500-1000 or HA gp 41.

15. The emulsion according to claim 9, wherein the hydrophilic polymer is selected from the group consisting of polyoxyethylene, polyethyloxazoline and polyethyleneglycol.
16. The emulsion according to claim 11, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, diacetylenic phospholipid and derivatives thereof and the non-ionic surface active agent is selected from the group consisting of poloxamer, sorbitan ester, polyoxyethylene-sorbitan fat acid ester and polyoxyethylene ethers.
17. The emulsion according to claim 11, wherein the emulsifier is selected from the group consisting of 1,2-dioleoyl-sn-3-phosphatidylethanolamine, diolein, fatty alcohol, cholesterol and bile salt.
18. The solid lipid nanoparticle according to claim 2, further comprising 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.
19. The solid lipid nanoparticle according to claims 2, wherein the emulsifier further comprises a phospholipid or a non-ionic surfactant.
20. The solid lipid nanoparticle according to claim 2, wherein the cationic surfactant is selected from the group comprising of, 1,2-dimyristoyl-3-trimethylammonium-propane, 1,2-dipalmitoyl-3-trimethylammonium-propane, 1,2-distearoyl-3-trimethylammonium-propane, 1,2-dioleoyl-3-trimethylammonium-propane, 1,2-dimyristoyl-3-dimethylammonium-propane, 1,2-dipalmitoyl-3-dimethylammonium-propane, 1,2-dilauroyl-3-dimethylammonium-propane, 1,2-distearoyl-3-dimethylammonium-propane, dimethyldioctadecylammonium chloride,

N-[1-(1,2-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride, 1,2-dioleoyl-3-ethylphosphocholine, and other cationic phospholipid.

21. The solid lipid nanoparticle according to claim 2, further comprising of  
5 glycerol or fusogenic peptides.
22. The solid lipid nanoparticle according to claim 21, wherein the fusogenic peptide is polyethylene glycol of MW 500-1000 or HA gp 41.
- 10 23. The solid lipid nanoparticle according to claim 18, wherein the hydrophilic polymer is selected from the group consisting of polyoxyethylene, polyethyloxazoline and polyethyleneglycol.
24. The solid lipid nanoparticle according to claim 19, wherein the phospholipid is  
15 selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, diacetylenic phospholipid and derivatives thereof and the non-ionic surface active agent is selected from the group consisting of a poloxamer, sorbitan ester, polyoxyethylene-sorbitan fat acid ester and polyoxyethylene ethers.
- 20 25. The solid lipid nanoparticle according to claim 2, wherein the emulsifier is selected from the group consisting of 1,2-dioleoyl-sn-3-phosphatidylethanolamine, diolein, fatty alcohol, cholesterol and bile salt.
- 25 26. The emulsion loaded with a drug according to claim 3, further comprising 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.
27. The emulsion loaded with a drug according to claim 3, wherein the emulsifier  
30 further comprises a phospholipid and a non-ionic surfactant.
28. The emulsion loaded with a drug according to claim 27, wherein the emulsifier is a phospholipid.



29. The emulsion loaded with a drug according to claim 27, wherein the surfactant is selected from the group consisting of, cationic, neutral and anionic phospholipids.
- 5 30. The emulsion loaded with a drug according to claim 3, further comprising glycerol, fusogenic peptides or proteins.
31. The emulsion loaded with a drug according to claim 30, wherein the fusogenic peptide is polyethylene glycol of MW 500-1000 or HA gp 41.
- 10 32. The emulsion loaded with a drug according to claim 26, wherein the hydrophilic polymer is selected from the group consisting of polyoxyethylene, polyethyloxazoline and polyethyleneglycol.
- 15 33. The emulsion loaded with a drug according to claim 28, wherein the phospholipid is selected from a group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, diacetylenic phospholipid and derivatives and the non-ionic surface active agent is selected from a group comprising of a poloxamer, sorbitan ester, polyoxyethylene-sorbitan fat acid ester and polyoxyethylene ethers.
- 20 34. The emulsion loaded with a drug according to claim 3, wherein the emulsifier is selected from a group consisting of 1,2-dioleoyl-sn-3-phosphatidylethanolamine, diolein, fatty alcohol, cholesterol and bile salt.
- 25 35. The emulsion loaded with a drug according to claim 3, wherein the lipophilic or amphiphilic drug is selected from the group consisting of antivirals, steroidal anti-inflammatory drugs, non-steroidal anti-inflammatory drugs, antibiotics, antifungals, vitamins, hormones, retinoic acid, prostaglandins, prostacyclins, anticancer drugs, antimetabolitic drugs, miotics, cholinergics, adrenergic antagonists, anticonvulsants, antianxiety agents, major tranquilizers, antidepressants, anesthetics, analgesics, anabolic steroids, estrogens, progesterones, glycosaminoglycans, polynucleotides,
- 30

immunosuppressants and immunostimulants.

36. The emulsion loaded with a drug according to claim 35, wherein the antiviral is diclofenac sodium or diclofenamic acid.
- 5 37. The emulsion loaded with a drug according to claim 35, wherein the immunosuppressant is cyclosporin A.
- 10 38. The emulsion loaded with a drug according to claim 3, wherein the lipid emulsion further comprises a hydrophilic drug in its aqueous phase.
39. The solid lipid nanoparticle loaded with a drug according to claim 4, further comprising 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.
- 15 40. The solid lipid nanoparticle loaded with a drug according to claim 4, wherein the emulsifier further comprises a phospholipid or a non-ionic surfactant.
41. The solid lipid nanoparticle loaded with a drug according to claim 40, wherein the emulsifier is a phospholipid.
- 20 42. The solid lipid nanoparticle loaded with a drug according to claim 41, wherein the phospholipid is selected from the group consisting of cationic, neutral and anionic phospholipid.
- 25 43. The solid lipid nanoparticle loaded with a drug according to claim 4, further comprising of a glycerol or fusogenic peptides or proteins.
44. The solid lipid nanoparticle loaded with a drug according to claim 43, wherein fusogenic peptide is polyethylene glycol of MW 500-1000 or HA gp 41.
- 30 45. The solid lipid nanoparticle loaded with a drug according to claim 39, wherein the hydrophilic polymer is selected from the group consisting of polyoxyethylene, polyethyloxazoline and polyethyleneglycol.

46. The solid lipid nanoparticle loaded with a drug according to claim 41, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, diacetylenic phospholipid and derivatives thereof and the non-ionic surface active agent is selected from the group consisting of a poloxamer, sorbitan ester, polyoxyethylene-sorbitan fat acid ester and polyoxyethylene ethers.
47. The solid lipid nanoparticle loaded with a drug according to claim 4, wherein the emulsifier is selected from the group consisting of 1,2-dioleoyl-sn-3-phosphatidylethanolamine, diolein, fatty alcohol, cholesterol and bile salt.
48. The solid lipid nanoparticle loaded with a drug according to claim 4, wherein the lipophilic or amphiphilic drug is selected from the group consisting of antivirals, steroidal anti-inflammatory drugs, non-steroidal anti-inflammatory drugs, antibiotics, antifungals, vitamins, hormones, retinoic acid, prostaglandins, prostacyclins, anticancer drugs, antimetabolitic drugs, miotics, cholinergics, adrenergic antagonists, anticonvulsants, antianxiety agents, major tranquilizers, antidepressants, anesthetics, analgesics, anabolic steroids, estrogens, progesterones, glycosaminoglycans, polynucleotides, immunosuppressants and immunostimulants.
49. The solid lipid nanoparticle loaded with a drug according to claim 48, wherein the antiviral is diclofenac sodium or diclofenamic acid.
50. The solid lipid nanoparticle loaded with a drug according to claim 49, wherein the immunosuppressant is cyclosporin A.
51. The solid lipid nanoparticle loaded with a drug according to claim 4, wherein the solid lipid nanoparticle further comprises a hydrophilic drug in its aqueous phase.
52. A complex of the emulsion according to claim 1 and a biologically active material selected from the group consisting of DNA, ribonucleic acid,

antisense nucleic acid, ribosome, polynucleotide, oligonucleotide, and other pharmaceutical drugs.

53. The complex according to claim 52, further comprising glycolipid, lipopeptide,  
5 antibody, ligand for receptors or viral protein to target specific cells or organs.
54. The complex according to claims 52 or 53, further comprising protamine sulfate, histone or cationic polymer.
- 10 55. The complex according to claim 54, wherein cationic polymer is polylysine.
56. The complex according to claim 52, further comprising monovalent or multivalent salt.
- 15 57. The complex according to claims 53, wherein the cell is selected from the group consisting of white blood cells, fibroblasts, cancer cells, cells infected with virus, epithelial cells, endothelial cells, muscle cells, liver cells, endocrine cells, neural cells, dermal cells, germ cells, oocytes, sperms, hematopoietic cells, fetal cells, M cells, Langerhans islet cells, macrophages, plant cells,  
20 animal cells, and immortalized cell lines.
58. The complex according to claim 52, wherein the complex is transferred to cells via intravenous, intramuscular, intratracheal, intranasal, subcutaneous, parenteral or topical administration or through direct administration to a  
25 specific organ.
59. A method of using the emulsion according to claim 1 to deliver DNA or biologically active materials to target cells.
- 30 60. The complex according to claim 52, further comprising lipophilic or amphiphilic drug in an oil phase.
61. The complex according to claims 60, wherein the drug is an anticancer drug.

62. A complex of the solid lipid nanoparticle according to claim 2 and a biologically active material complex selected from the group consisting of DNA, ribonucleic acid, antisense nucleic acid, ribosome, polynucleotide, oligonucleotide, or other pharmaceutical drugs.
- 5
63. The complex according to claim 62, further comprising glycolipid, lipopeptide, antibody, ligand for receptors or viral protein to target specific cells or organs.
64. The complex according to claims 62 or 63, further comprising protamine sulfate, histone or cationic polymer.
- 10
65. The complex according to claims 64, wherein the cationic polymer is polylysine.
- 15
66. The complex according to claims 62, further comprising monovalent or multivalent salt.
67. The complex according to claims 63, wherein the cell is selected from the group consisting of white blood cells, fibroblasts, cancer cells, cells infected with virus, epithelial cells, endothelial cells, muscle cells, liver cells, endocrine cells, neural cells, dermal cells, germ cells, oocytes, sperms, hematopoietic cells, fetal cells, M cells, Langerhans islet cells, macrophages, plant cells, animal cells, and immortalized cell lines.
- 20
68. The complex according to claim 62, wherein the complex is transferred to cells via intravenous, intramuscular, intratracheal, intranasal, subcutaneous, parenteral or topical administration or through direct administration to a specific organ.
- 25
69. The complex according to claim 52, further comprising a lipophilic or amphiphilic drug in the fat.
- 30
70. The complex according to claims 69, wherein the drug is an anticancer drug.

71. The method according to claim 5, wherein the aqueous phase further comprises of 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.
72. The method according to claim 6, wherein the aqueous phase further  
5 comprises of 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.
73. The method according to claim 7, wherein the aqueous phase further comprises of 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.
- 10 74. The method according to claim 8, wherein the aqueous phase further comprises of 0.01-10% of a hydrophilic polymer or hydrophilic polymeric lipid.
75. The method according to claims 5, wherein the emulsifier is added in an oil phase instead of in an aqueous phase.
- 15 76. The method according to claims 6, wherein the emulsifier is added in a melted fat instead of in the aqueous phase.
77. The method of according to claims 7, wherein the emulsifier is added in the  
20 oil phase instead of in an aqueous phase.
78. The method according to claims 8, wherein the emulsifier is added in the melted fat instead of in the aqueous phase.

1/25

FIG.1

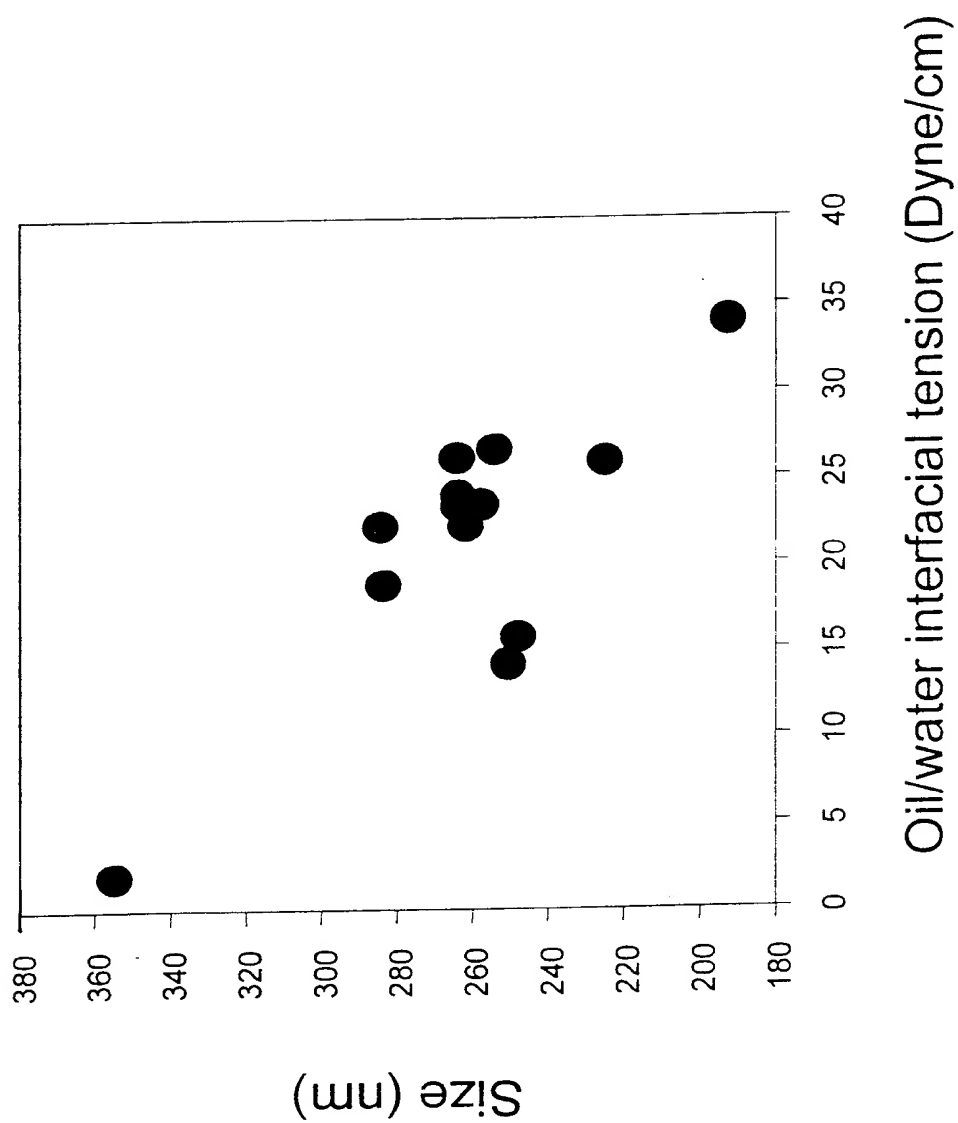
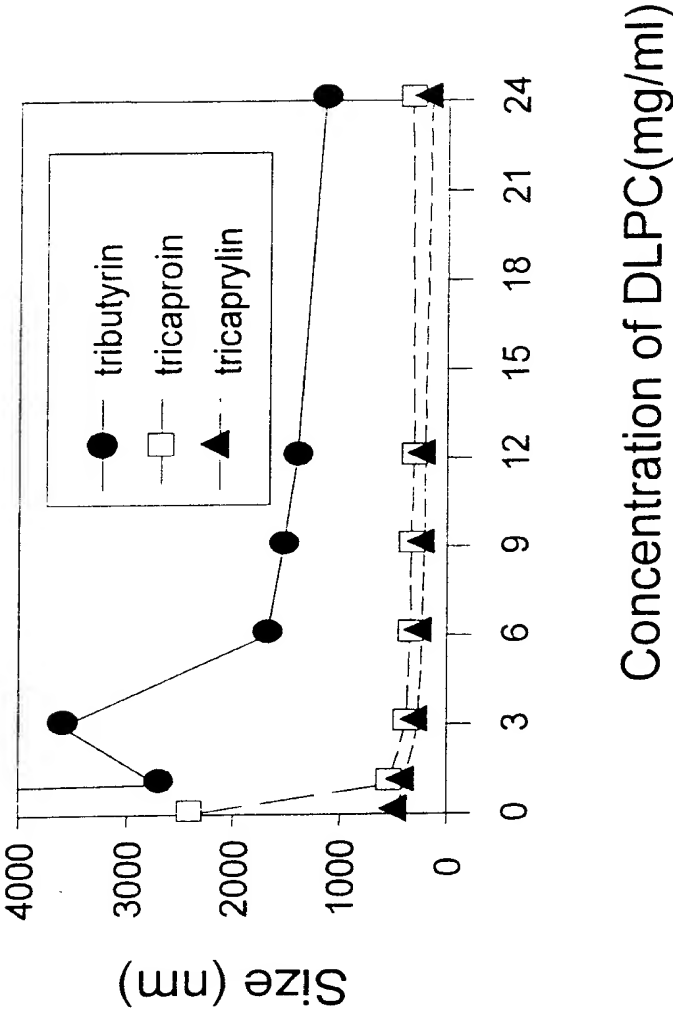


FIG. 2A





3/25

FIG. 2B

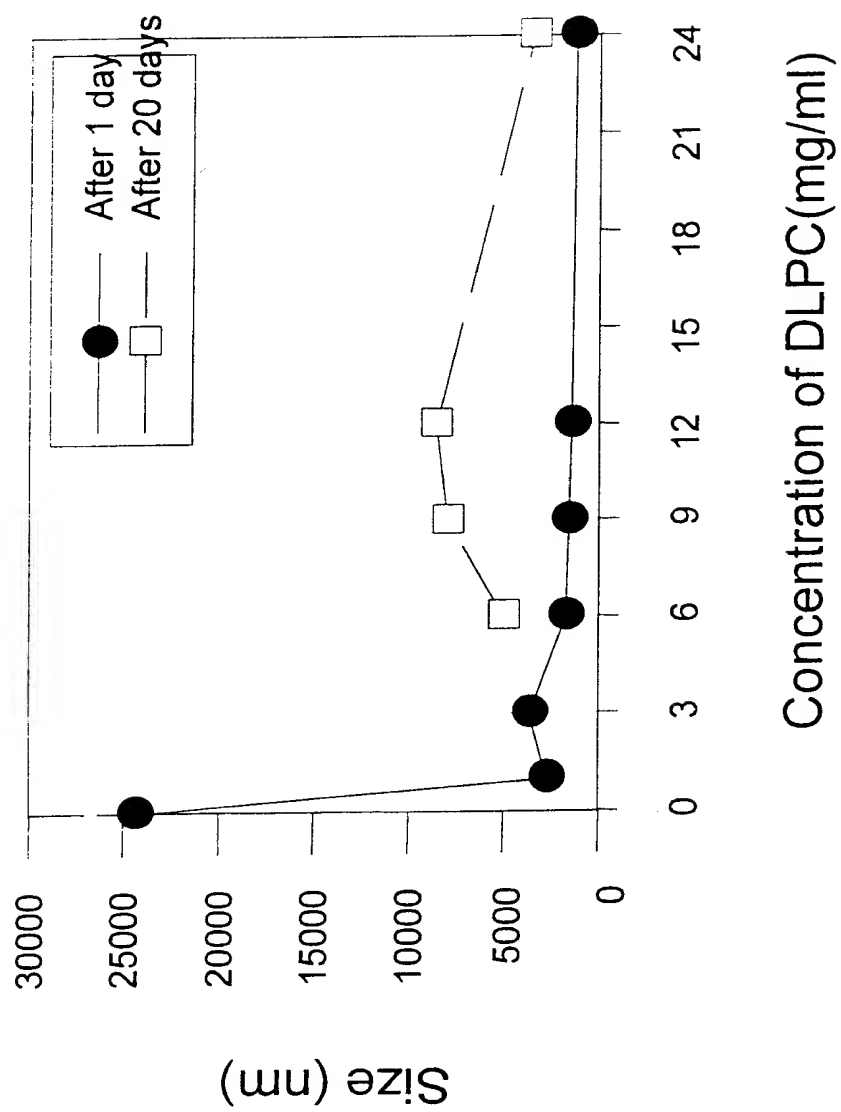
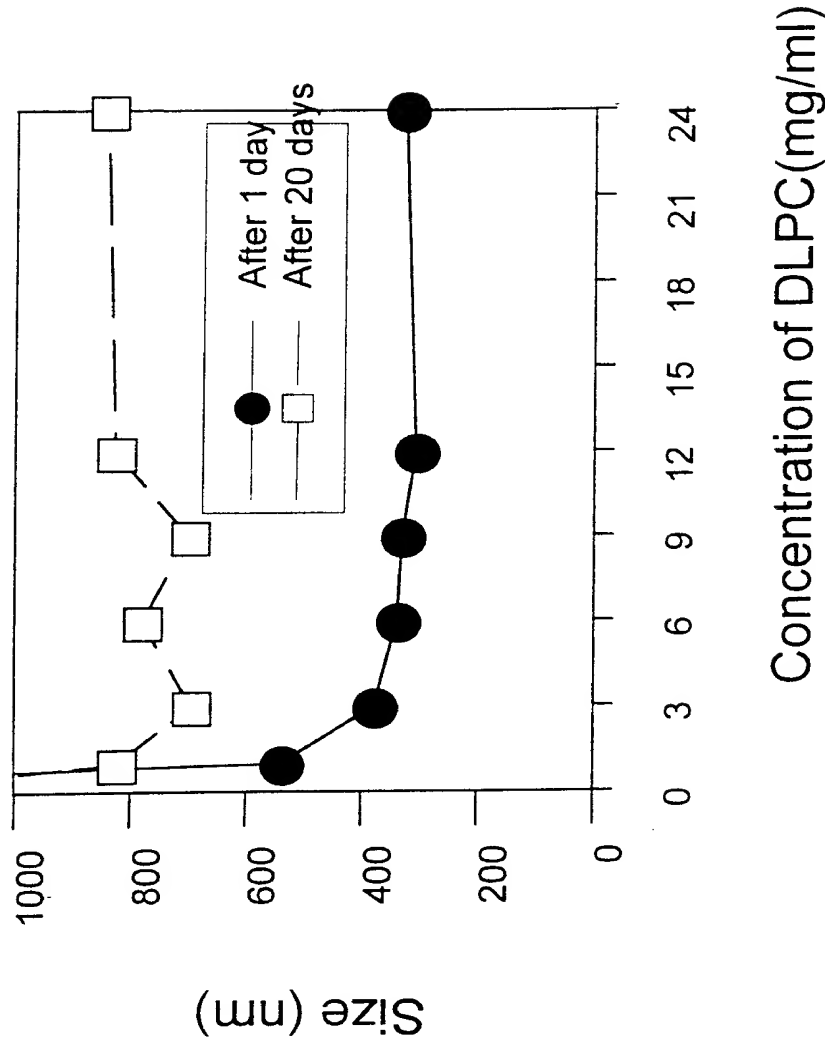
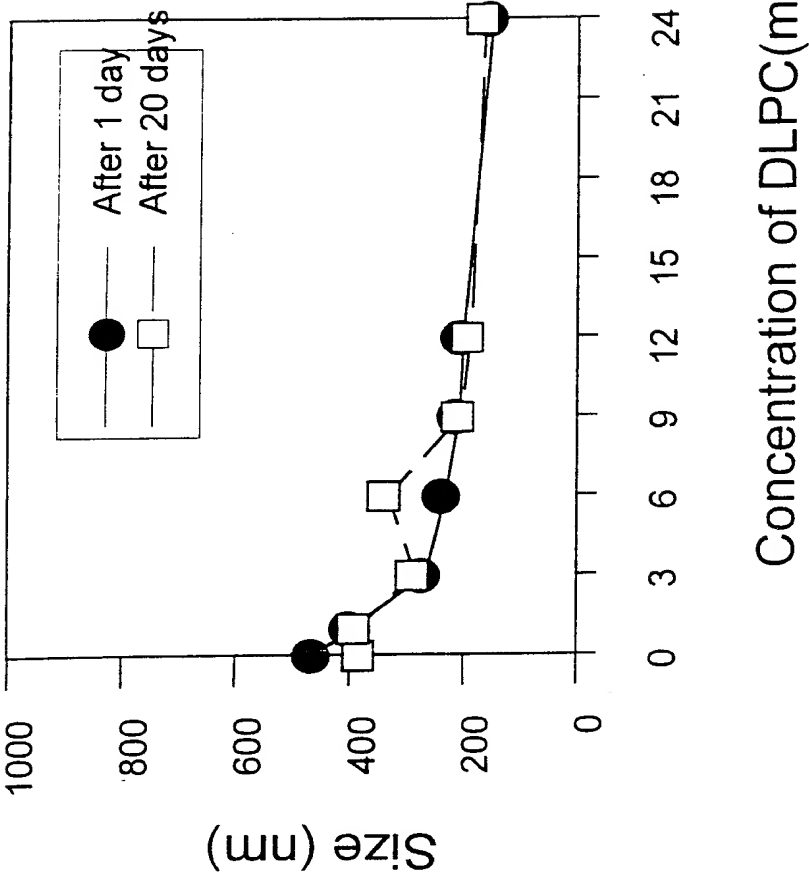


FIG.2C



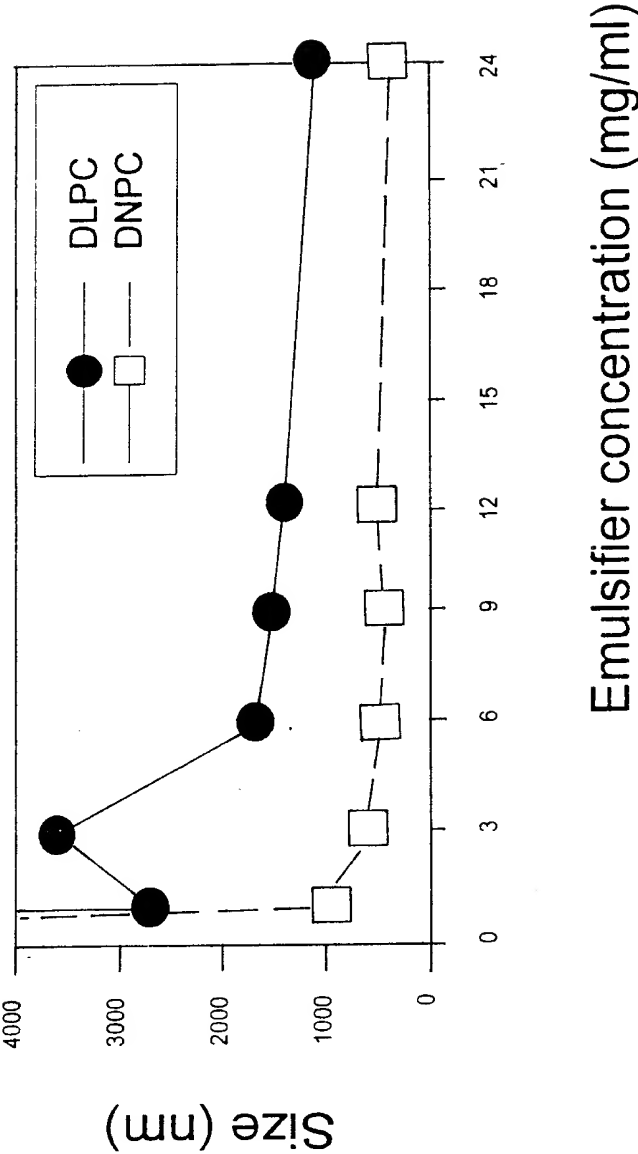
5/25

FIG. 2D



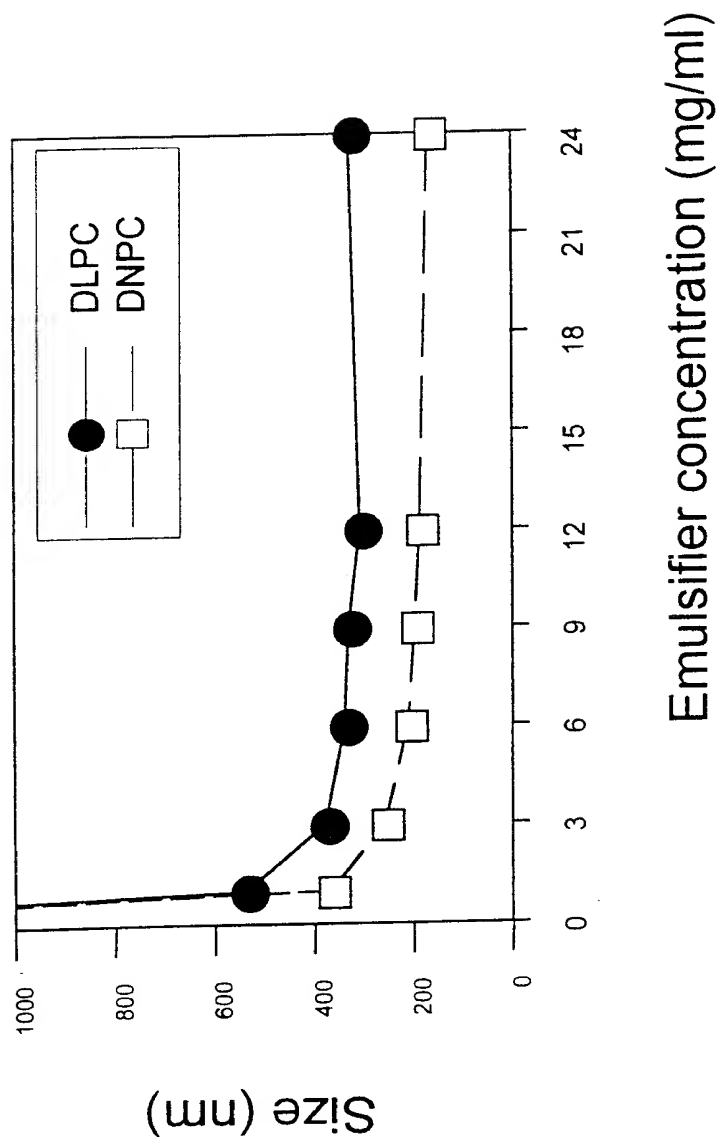
6/25

FIG.3A



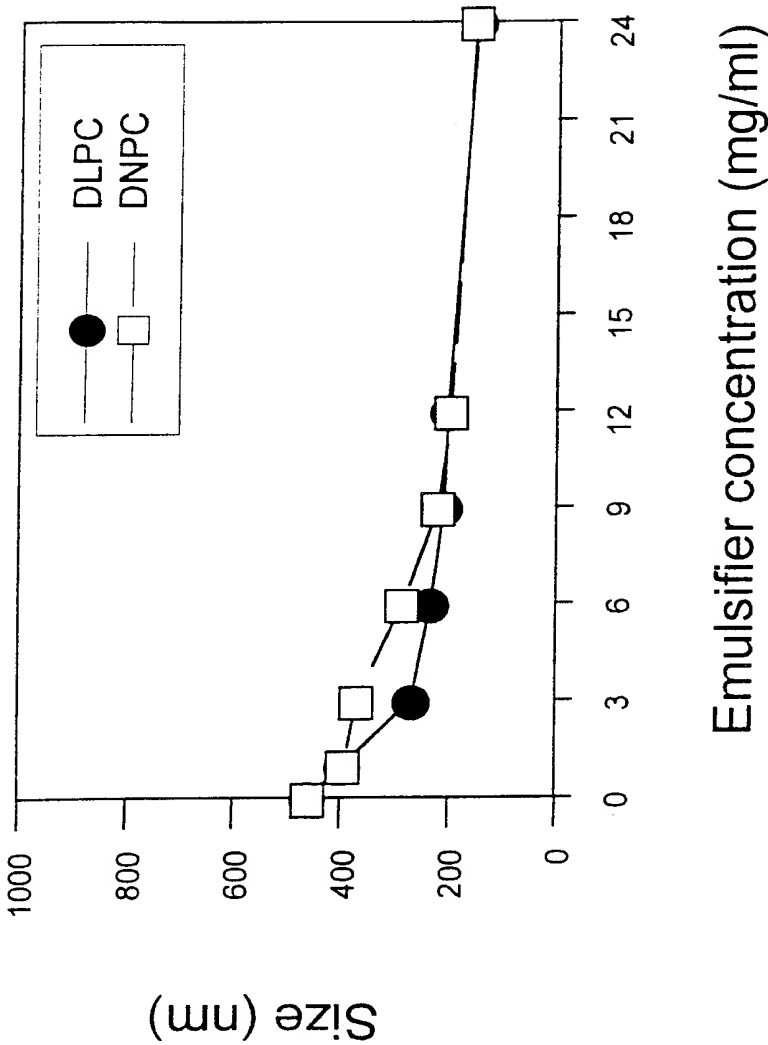
7/25

FIG. 3B



8/25

FIG.3C



9/25

FIG.4

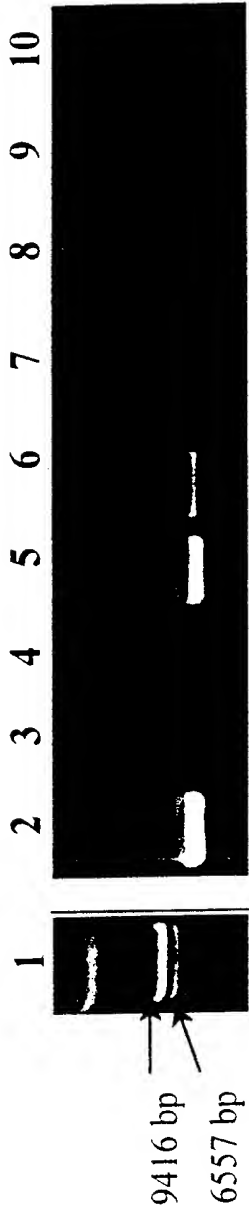
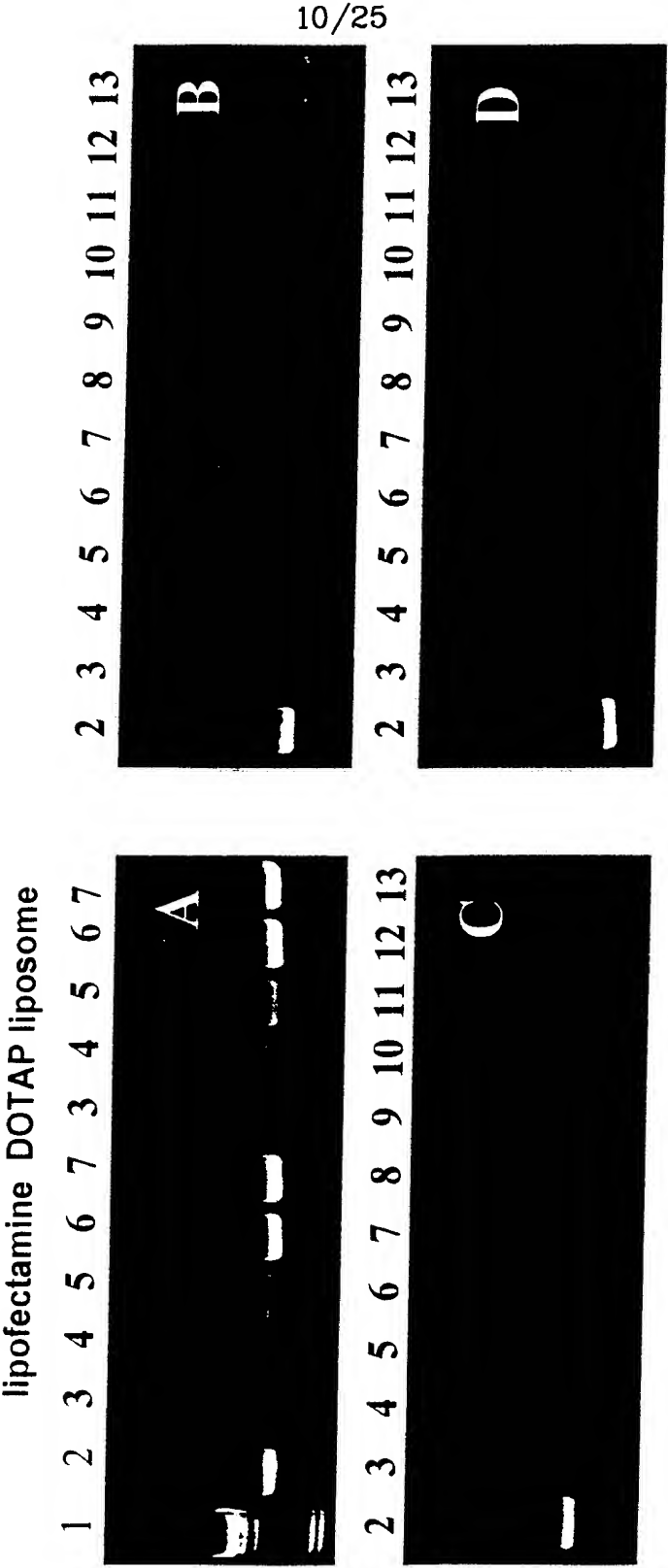


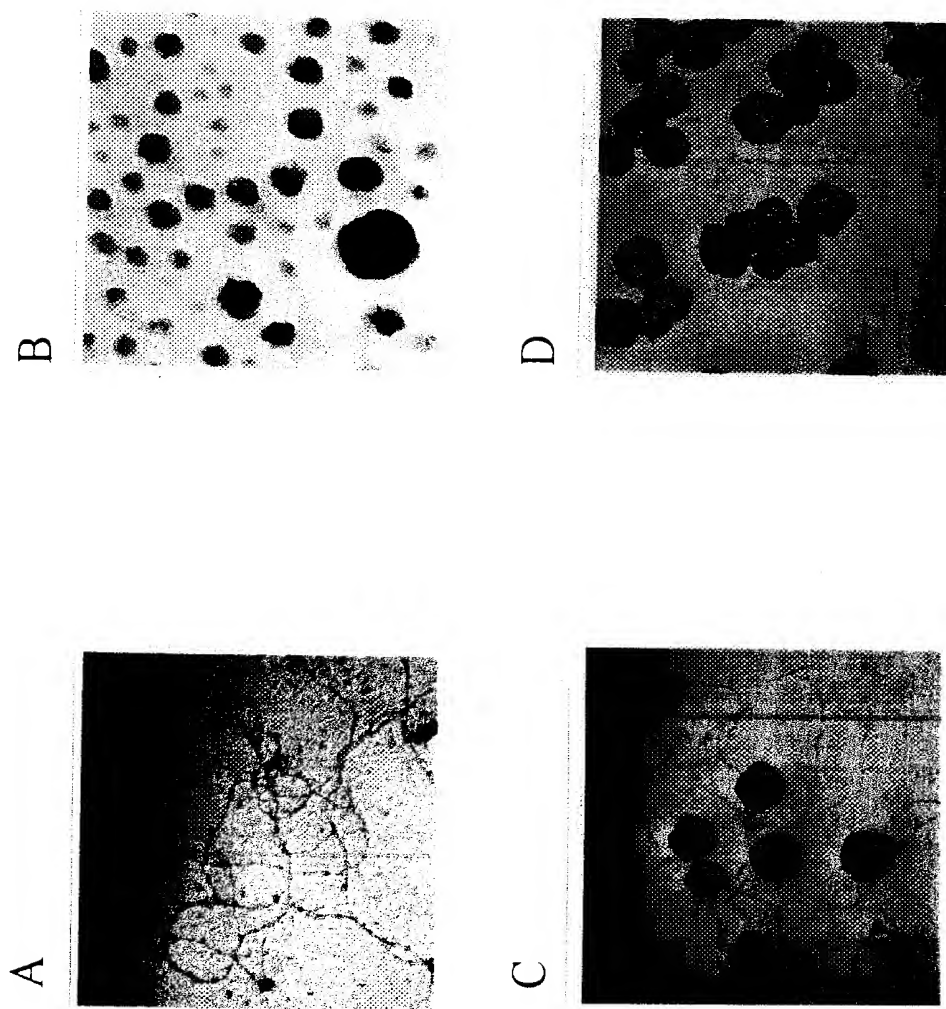
FIG.5





11/25

FIG. 6



12/25

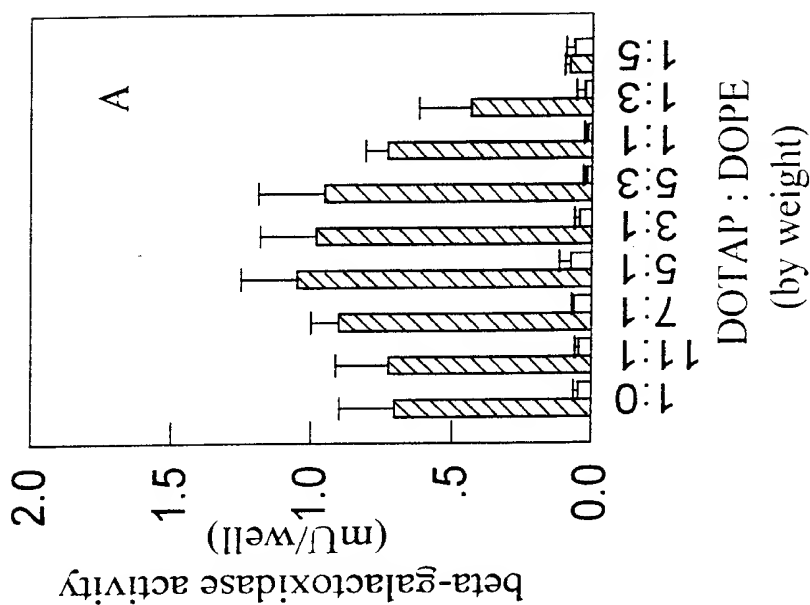
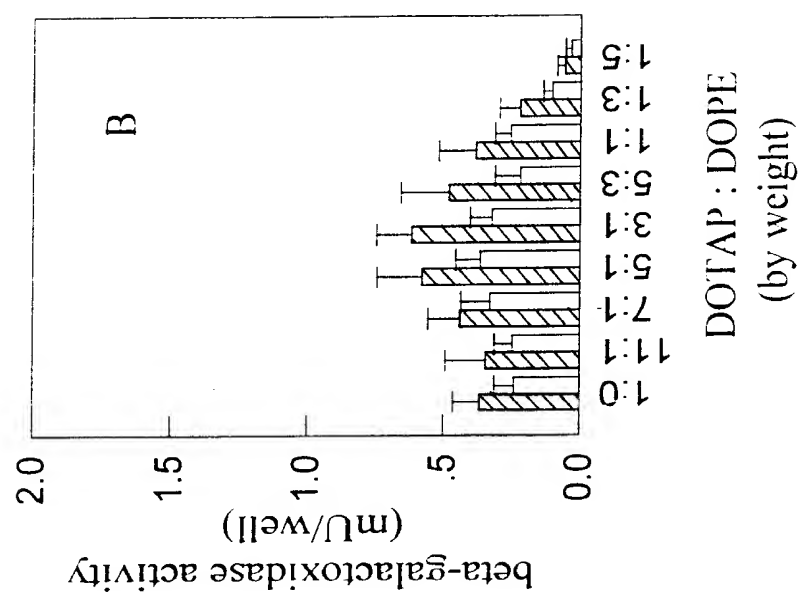
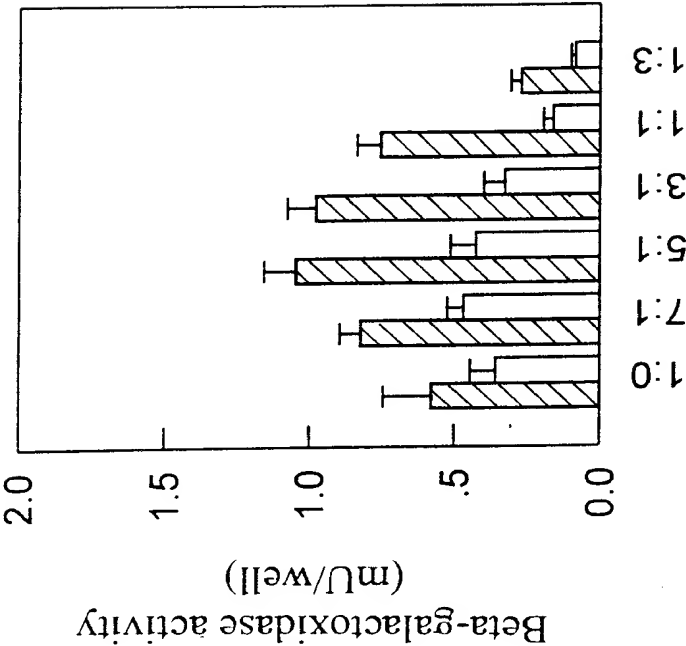


FIG. 7

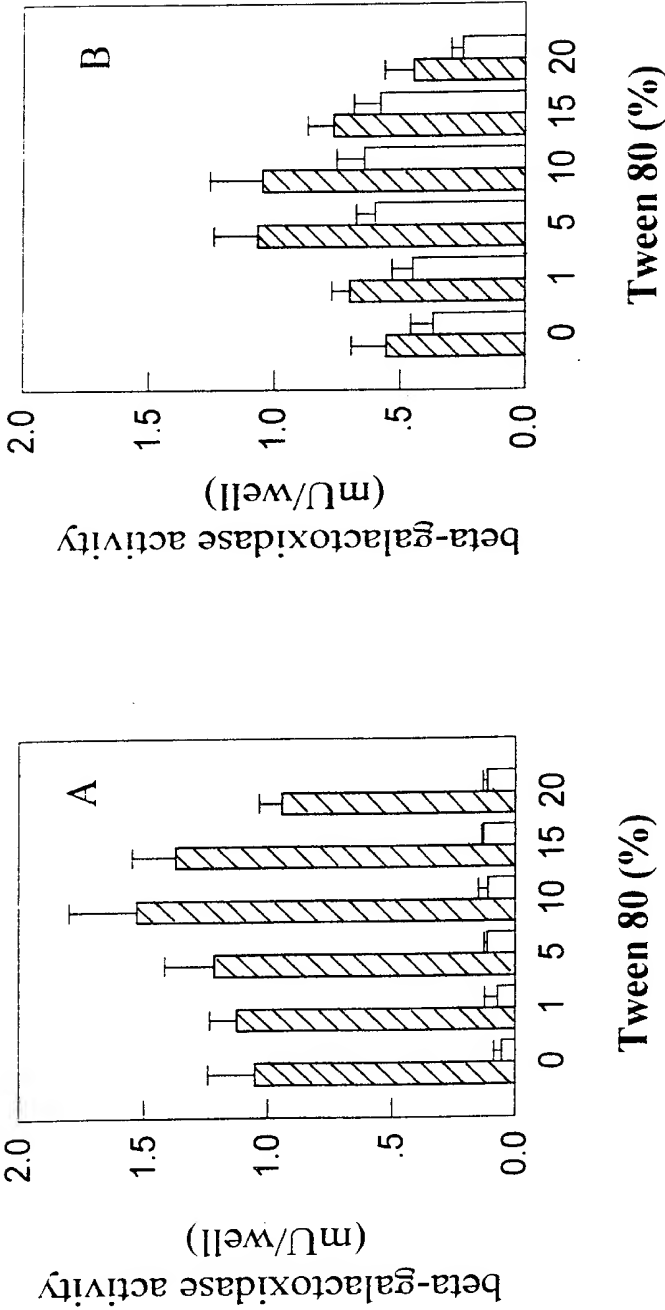
FIG.8



(DOTAP / DOPE 5/1) : DIOLEIN  
(by weight)

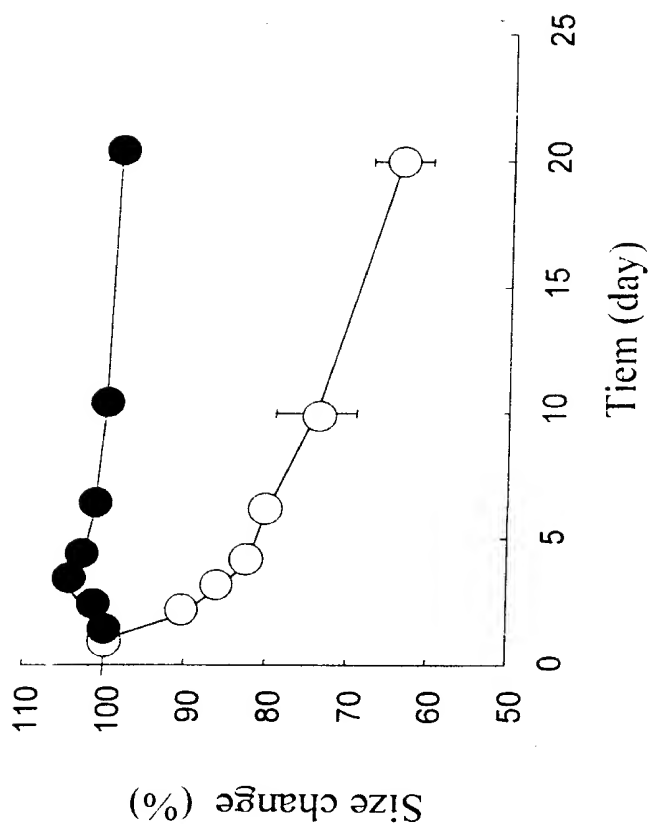
14/25

FIG. 9



15/25

FIG.10



16/25

FIG.11

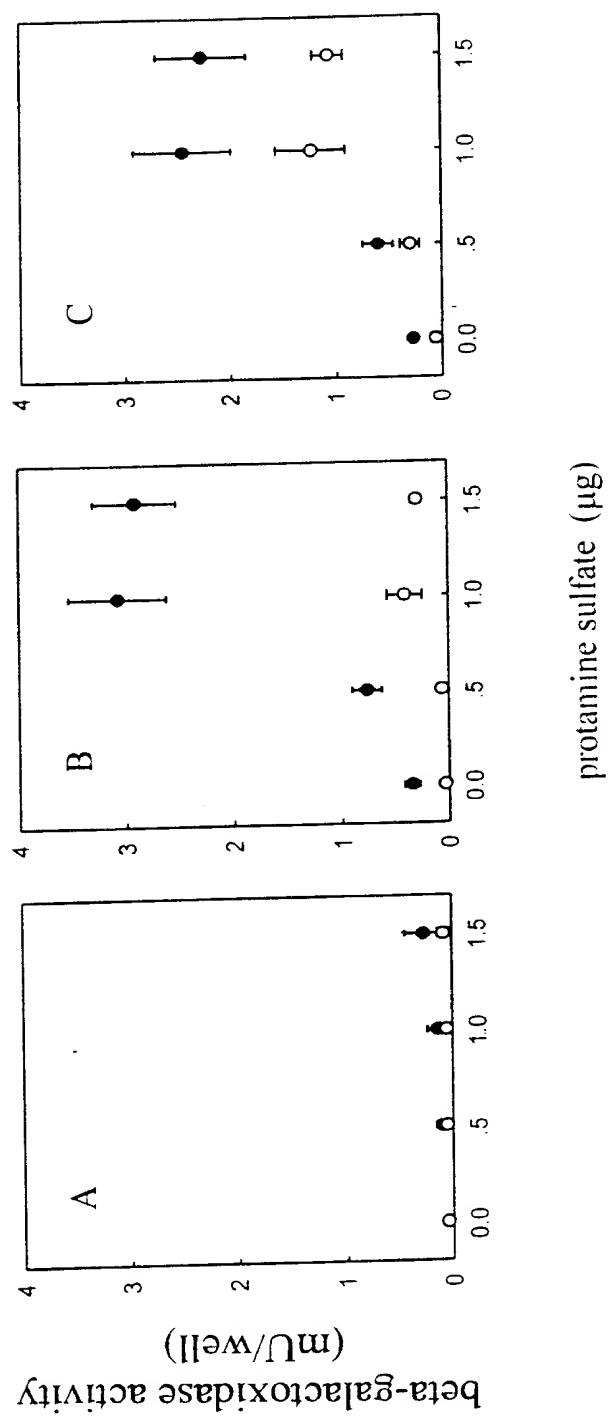
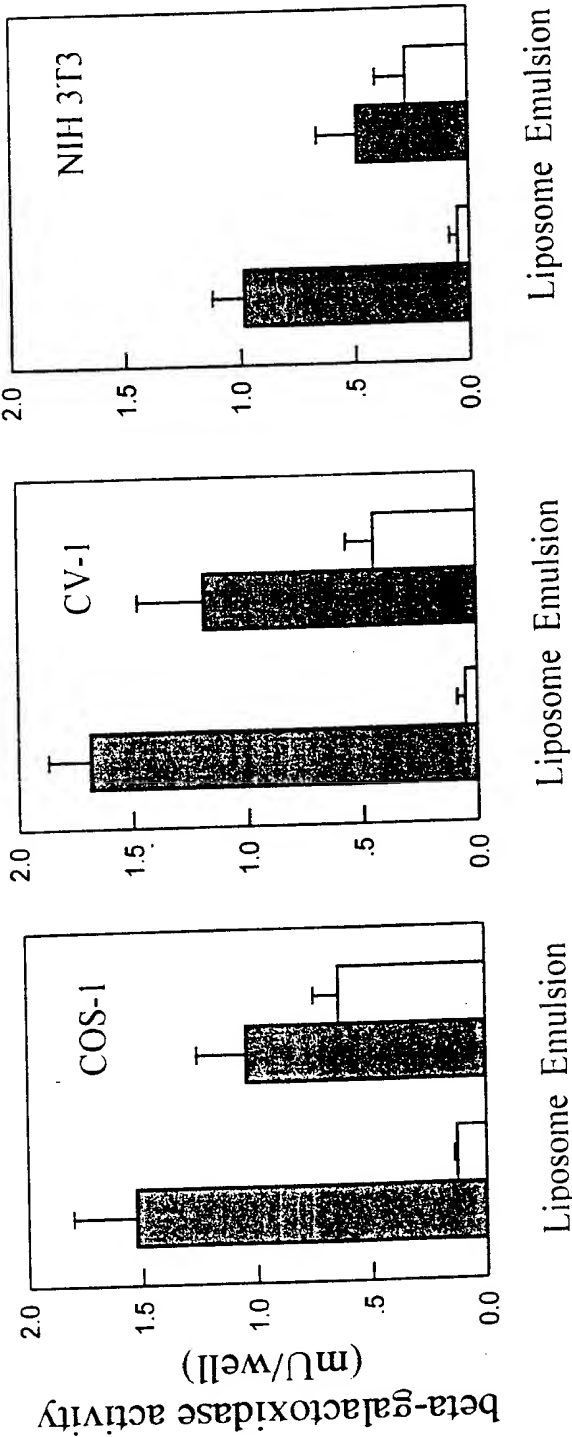
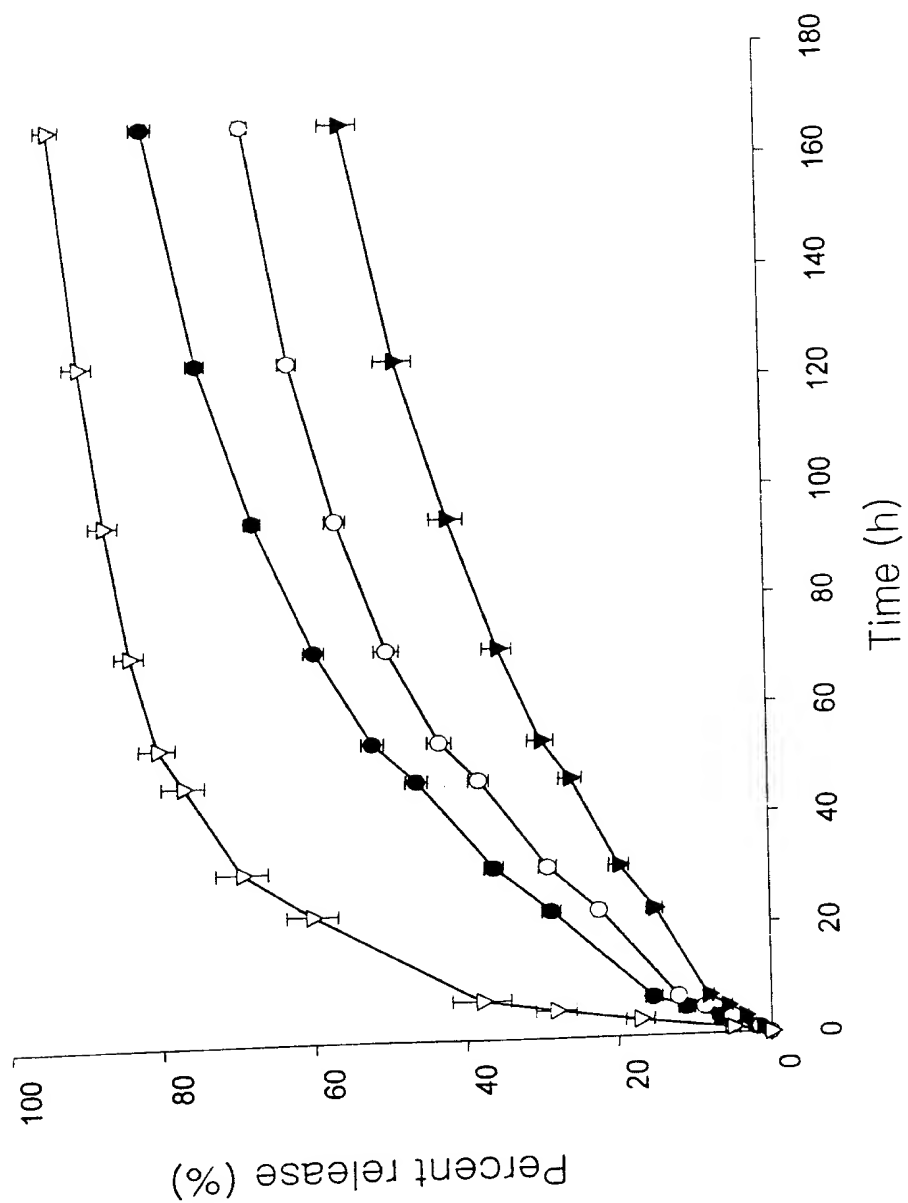


FIG.12



18/25

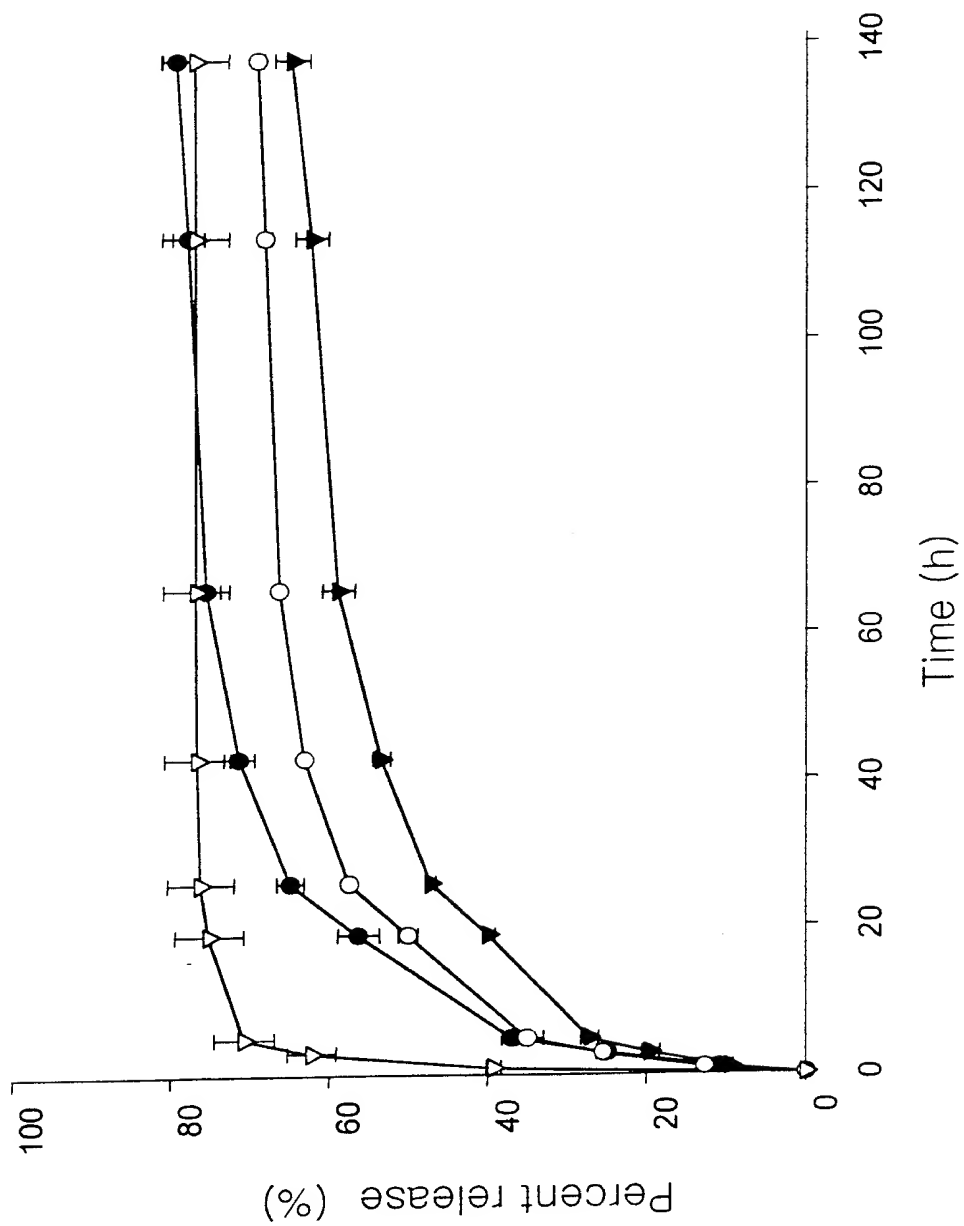
FIG.13





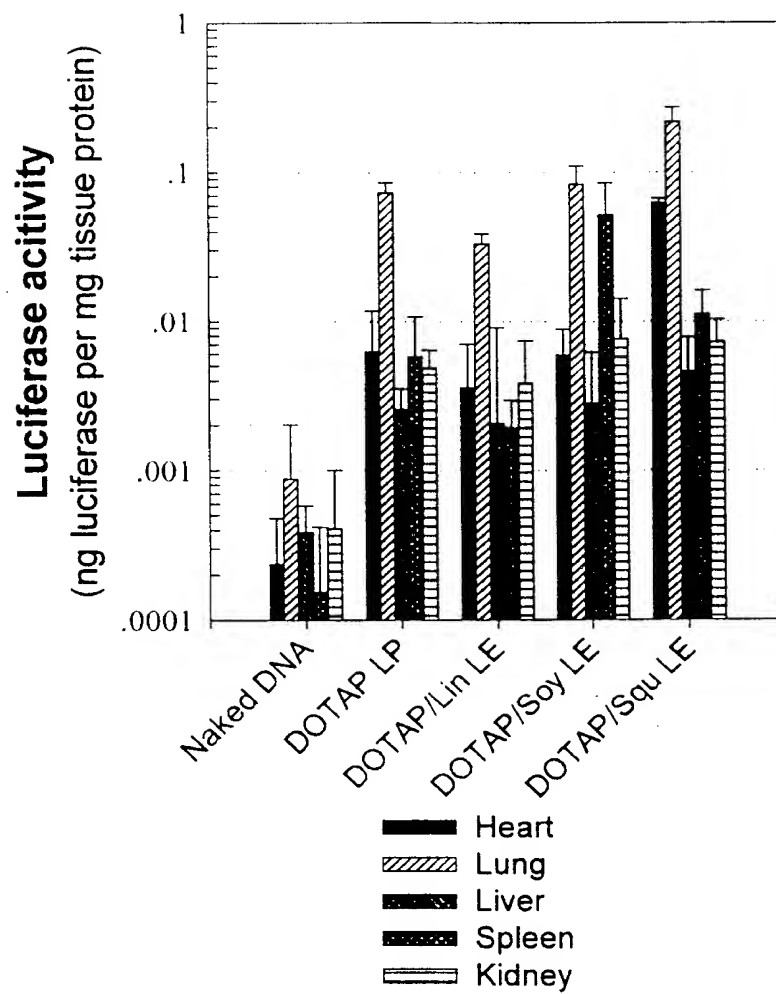
19/25

FIG.14



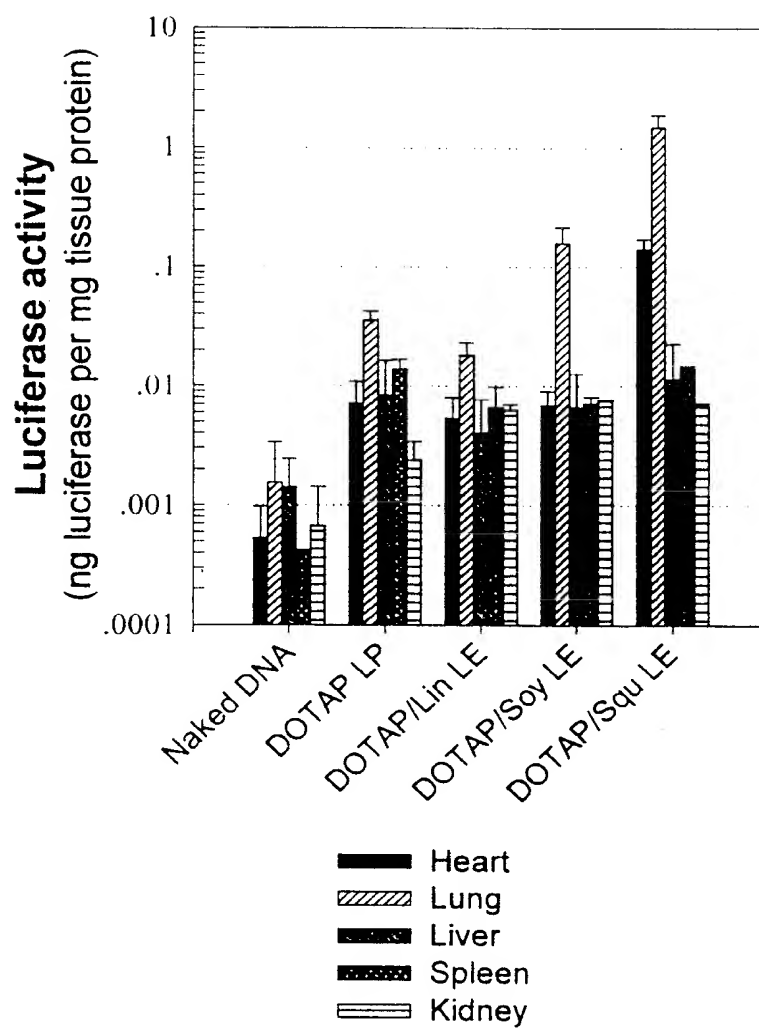
20/25

FIG.15



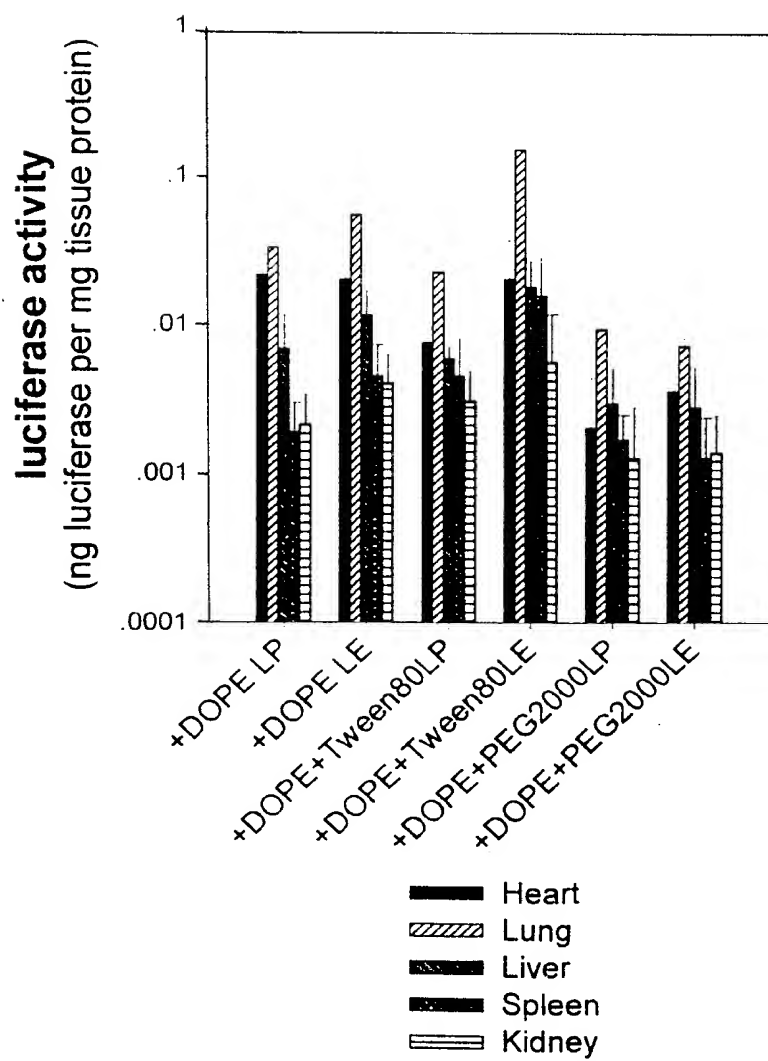
21/25

FIG.16



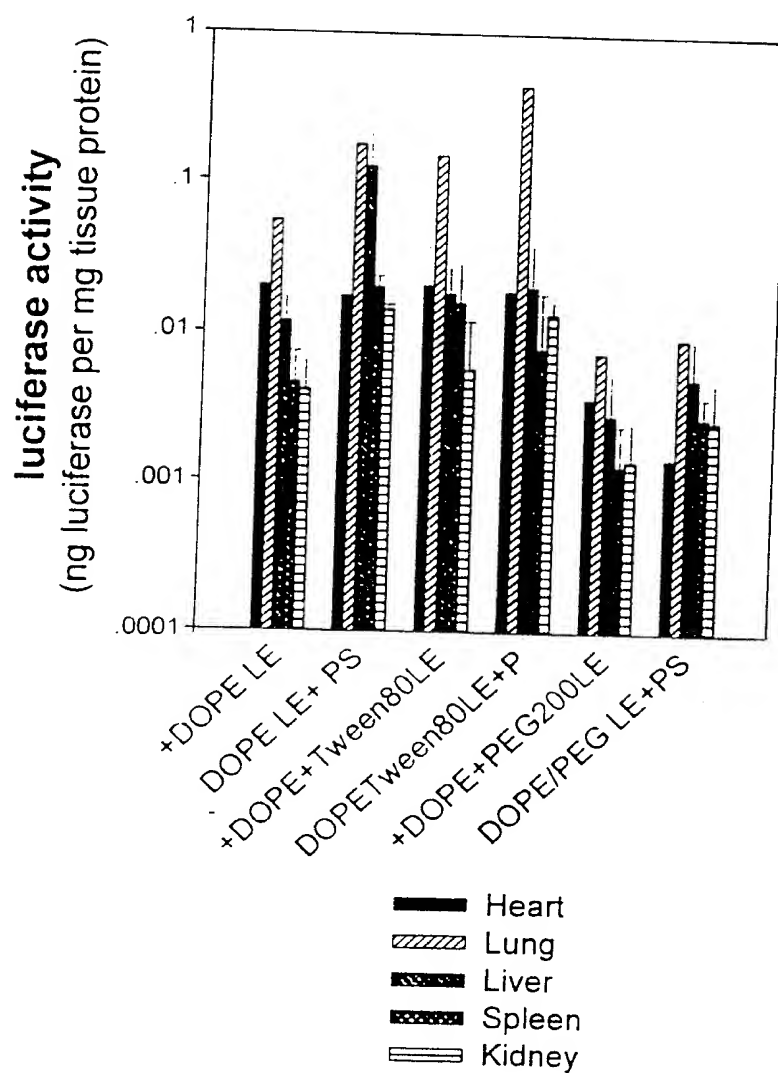
22/25

FIG.17



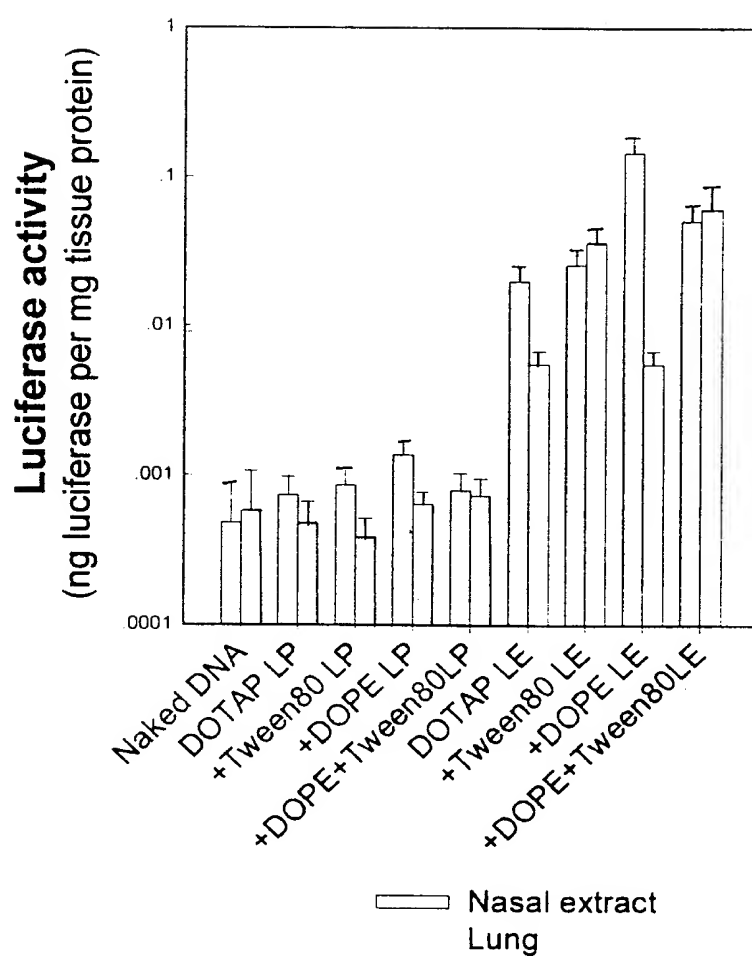
23/25

FIG.18



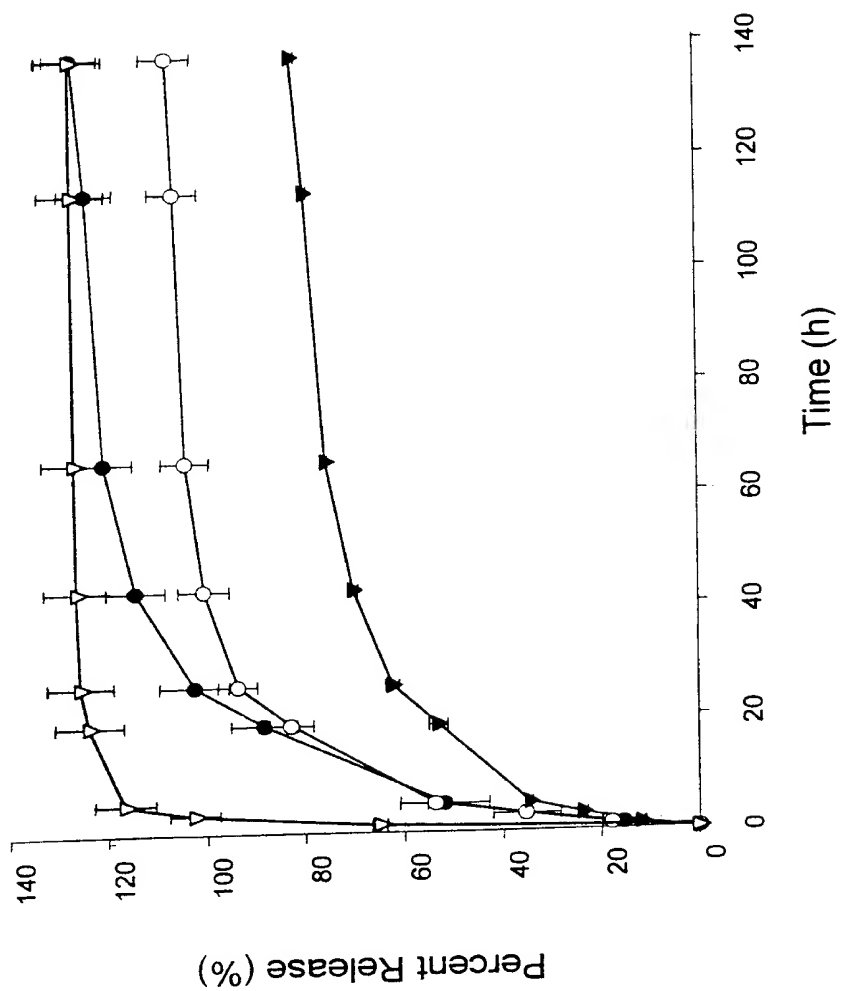
24/25

FIG.19



25/25

FIG. 20



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/KR 99/00414

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>7</sup>: A 61 K 9/107

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>7</sup>: A 61 K 9/107

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/25917 A1 (BAUSCH & LOMB INCORPORATED) 29 August 1996 (29.08.96) claims 1,19,23,25,28.	1,13
X	WO 93/00160 A1 (EMORY UNIVERSITY) 07 January 1993 (07.01.93) page 18, line 38 - page 20, line 7; page 21; tables 1-4; claims 1,5,8.	3,26,35
X	EP 0490053 A1 (WELLA AKTIENGESELLSCHAFT) 17 June 1992 (17.06.92) claims 1,5,6,7.	1
X	JP 03-044314 A (NIPPON PETROCHEMICALS CO LTD) 1991-02-26 (abstract) [online] [retrieved on 1991-11-08]. Retrieved from EPO PAJ Database.	1
X	JP 06-219940 A (SHISEIDO CO. LTD.) 1994-08-09 (abstract) [online] [retrieved on 1999-11-08]. Retrieved from EPO PAJ Database	1,3,7,52

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

„A“ document defining the general state of the art which is not considered to be of particular relevance

„E“ earlier application or patent but published on or after the international filing date

„L“ document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

„O“ document referring to an oral disclosure, use, exhibition or other means

„P“ document published prior to the international filing date but later than the priority date claimed

„T“ later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

„X“ document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

„Y“ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

„&“ document member of the same patent family

Date of the actual completion of the international search

08 November 1999 (08.11.99)

Date of mailing of the international search report

06 December 1999 (06.12.99)

Name and mailing address of the ISA/AT  
Austrian Patent Office  
Kohlmarkt 8-10; A-1014 Vienna  
Facsimile No. 1/53424/200

Authorized officer

Mosser

Telephone No. 1/53424/437



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/KR 99/00414

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim No.: 58  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 58 concerns the treatment of the human or animal body by therapy (see PCT Rule 39.1 (iv) the search was carried out and based on the alleged effects.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. claims 1, 3, 5, 7, 9-17, 26-38, 52-61, 71, 73, 75 and 77 concern lipid emulsions comprising non-triglyceride oil.
2. claims 2, 4, 6, 8, 18-25, 39-51, 62-70, 72, 74, 76 and 78 concern solid lipid nanoparticles comprising fat of triglycerides.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:  
1, 3, 5, 7, 9-17, 26-38, 52-61, 71, 73, 75, 77.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 99/00414

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche		Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WD A1	9625917	29-08-1996	AU A1 48683/96 CA AA 2213517 EP A1 810852 US A 5759557 US A 5759558	11-09-1996 29-08-1996 10-12-1997 02-06-1998 02-06-1998
WD A1	9300160	07-01-1993	AT E 165749 AU A1 23158/92 CA AA 2112356 CN A 1073889 CN A 1096224 CN B 1042198 DE C0 69225404 DE T2 69225404 EP A1 591452 EP A4 591452 EP B1 591452 ES T3 2116340 HK A1 1010450 IE B 67066 IL A0 102317 IL A0 107262 JP T2 6508555 US A 5885590	15-05-1998 25-01-1993 07-01-1993 07-07-1993 14-12-1994 24-02-1999 10-06-1998 17-12-1998 13-04-1994 20-07-1994 06-05-1998 16-07-1998 17-06-1999 21-02-1996 14-01-1993 25-01-1994 29-09-1994 23-03-1999
EE A	490053		keine - none - rien	
JP A2	3044314	26-02-1991	keine - none - rien	
JP A2	6219940	09-08-1994	keine - none - rien	